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Individual Specialization and Assortative Mating in Undifferentiated Populations

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Individual Specialization and Assortative Mating in Undifferentiated Populations

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Individual Specialization and Assortative Mating in Undifferentiated Populations

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Individual specialization occurs when individuals selectively consume a subset of their population's diet. Intraspecific diet variation can stabilize population and community dynamics, promote species coexistence, and increase ecosystem productivity. Ecological variation also provides the variability necessary for natural or sexual selection to act.

Individual threespine stickleback select different prey from a shared environment, and this variation is not simply a result of sex, size, or spatial heterogeneity. I use longitudinal observation of stickleback foraging microhabitat to support more commonly used cross-sectional metrics. Among recaptured individuals there were correlations between microhabitat use and functional morphology, and microhabitat use and long term dietary differences between individuals.

I quantify individual specialization across populations using cross-sectional sampling to understand how and why ecological variation may itself be variable. All populations showed significant individual specialization. Specialization varied between populations and this variation seems to be a long-term property of populations. Overall morphological variance was positively correlated with ecological variation.

Ecological variation, like all types of heritable variation, provides raw material for evolutionary change. For example, lacustrine populations of stickleback are commonly under disruptive selection due to intraspecific competition for prey resources. Speciation with gene flow may be driven by a combination of positive assortative mating and disruptive selection, particularly if selection and assortative mating act on the same trait. We present evidence that stickleback exhibit assortative mating by diet, using the isotopes of males and eggs within their nests. In concert with disruptive selection, this assortative mating should facilitate divergence. However, the population remains phenotypically unimodal, highlighting the fact that assortative mating and disruptive selection do not guarantee evolutionary divergence and speciation.

There are several not-mutually-exclusive mechanisms by which assortative mating by diet may occur in these populations, such as shared microhabitat preference among individuals of similar diet. Stable isotopes reveal diet differences between different nesting areas and among individuals using different nest habitat within a nesting area. Spatial segregation of diet types may generate some assortative mating, but is insufficient to explain the observed assortment strength. We therefore conclude that sticklebacks' diet-assortative mating arises primarily from behavioral preference rather than from spatial isolation.

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Chapter 1

Dissertation Summary

Many ecologically generalized populations are composed of relatively specialized individuals that selectively consume a subset of their population's diet, a phenomenon known as 'individual specialization' (Van Valen 1965; Roughgarden 1972; Grant and Price 1981; Smith and Skulason 1996; Wilson 1998; Bolnick et al. 2003). An individual's diet is determined by its (1) encounter rate with prey (e.g., microhabitat use; Holbrook and Schmitt 1992); (2) ability to detect prey (e.g., search images due to past experience; Persson 1985; Hughes and Croy 1993); (3) motivation to consume particular prey once encountered (e.g., selectivity; Werner 1974), and (4) ability to capture and consume the prey once a decision to attack has been made (e.g., biomechanical adaptations; Robinson 2000; Ferry-Graham et al. 2002). All of these factors depend on phenotypic characters that may exhibit genetic or environmental variance among individuals, resulting in among-individual diet variation.

Individual specialization can play important roles in both evolutionary and ecological dynamics (Saloniemi 1993; Doebeli 1996; Doebeli 1997; Ackermann and Doebeli 2004; Okuyama 2008; Bolnick et al. 2011; Schreiber et al. 2011), and is also of interest to behavioral ecologists studying behavioral syndromes or consistency of behavior (Dall et al. 2012). Emerging theory on the ecological consequences of individual specialization suggests that intraspecific diet variation can stabilize population and community dynamics, promote species coexistence, and increase ecosystem productivity (Fox and Kendall 2002; Doebeli 1996, 1997; Saloniemi 1993; Bolnick et al. 2011; Schreiber et al. 2011). Some of these predicted ecological consequences have been

tested experimentally, confirming that genetic variation promotes population persistence and stability and that the degree of morphological variance has strong effects on ecosystem primary productivity and prey community structure (e.g., Agashe 2009, Harmon et al. 2009, Ingram et al. 2011). Ecological variation provides the variability necessary for natural (Bolnick and Lau 2008) or sexual (Chapter 3) selection to act. While there has been growth in the quantification of individual specialization (called for by Bolnick et al. 2003, reviewed in Araújo et al. 2011), shifting focus away from the historical focus of simply testing the null hypothesis that individuals have the same niche, many fundamental questions about individual specialization have gone unanswered. In this dissertation I answer several important standing questions about individual specialization. I then go on to document one way individual specialization may affect evolutionary dynamics—through assortative mating between ecologically similar individuals.

The threespine stickleback (*Gasterosteus aculeatus*) is a model organism for the study of evolution, ecology, and behavior, and has been used extensively to study individual specialization in wild populations. Multiple mechanisms can contribute to this diet variation, including ecological sexual dimorphism, ontogenetic niche shifts, spatial heterogeneity, and phenotypic variation (including morphology and behavior). Dietary sexual dimorphism has been documented in stickleback for some populations (Reimchen and Nosil 2001a; Reimchen et al. 2008; Spoljaric and Reimchen 2008), but not others (Bolnick et al. 2008; Bolnick and Paull 2009). Ontogenetic niche shifts occur as well, but within adults we have found that diet (stomach contents or isotopes) is typically more strongly correlated with body shape than with size (Chapter 3; Bolnick and Paull 2009). Finally, diet variation in stickleback is not simply a result of coarse-grained variation in prey availability (i.e., at a scale greater than sticklebacks' daily cruising range while

foraging), because similar levels of diet variation are observed among fish held in a 10 m^2 enclosure that ensured equal access to prey (Svanbäck and Bolnick 2007). Thus, it is clear that individual stickleback select different subsets of prey from a shared environment, and that this variation is not simply a result of sex, size, or spatial heterogeneity.

In chapter two I use two studies to quantify individual specialization in stickleback. First, I use longitudinal observation of stickleback foraging microhabitat use to quantify individual specialization. Repeated sampling of individuals is considered the gold standard in quantifying ecological variation among individuals, but has rarely been used due to the labor-intensive methods employed. Longitudinal studies are particularly difficult to use in aquatic organisms that cannot easily be observed directly. On average, a pair of fish from our study were about 43% divergent in their relative use of alternative microhabitats, which corresponds closely to measurements of individual specialization based on stomach contents of recaptured individuals, which showed 46% divergence in their relative use of prey taxa. Among recaptured individuals there were also correlations between microhabitat use and functional morphology, and microhabitat use and stable isotope ratios (indicating long term dietary differences between individuals). In addition to supporting more commonly used cross-sectional metrics of individual specialization, this study reveals microhabitat partitioning across more dimensions than the classic distinction between benthic and limnetic feeding strategies commonly discussed in relation to lacustrine fish.

Second, I quantified individual specialization in 12 lacustrine populations of stickleback using cross-sectional sampling including gut contents, stable isotopes, and morphology to understand how and why ecological variation may itself be variable. The Niche Variation Hypothesis (NVH) is the most prominent hypothesis as to why

individual specialization may be more pronounced in some populations than in others (Van Valen 1965). The NVH states that niche expansion during ecological release occurs mainly through diet divergence among individuals, with correspondingly higher morphological variation (Van Valen 1965). Subsequent tests of the NVH has led to mixed support, with many studies failing to find a correlation between morphological variance and population niche width (reviewed by Bolnick et al 2007). However, these tests presuppose that morphological variance is a suitable proxy for ecological variation. Previous studies of stickleback have repeatedly detected among-individual variation in stomach contents, isotopes, and morphology, as well as correlations between these traits (Chapter 3; Svanbäck and Bolnick 2007; Araújo et al. 2008; Reimchen et al. 2008; Bolnick and Paull 2009; Bolnick et al. 2010; Matthews et al. 2010). It is also known that there is variation among populations in the amount of within-population morphological variance (Berner et al. 2010), but chapter two is the first direct test of whether the amount of ecological variance within populations is correlated with the amount of morphological variance.

All populations showed significant individual specialization. Specialization varied between populations and snap-shot gut content metrics and longer-term isotope based metrics of dietary variation were correlated among populations, suggesting this variation is a long-term property of populations. While there were significant general relationships between morphology and diet among populations, there were also significant differences among populations as indicated by interaction terms between morphology and population and three-way interactions between morphology, population, and sex. It is not surprising then that variance in single morphological traits did not correlate with the amount of individual specialization among populations. However, we found that overall

morphological variance was positively correlated with ecological variation, supporting an often assumed but rarely tested piece of the niche variation hypothesis.

Ecological variation, like all types of heritable variation, provides the raw material for evolutionary change. For example, lacustrine populations of stickleback are commonly under disruptive selection due to intraspecific competition for prey resources (Bolnick and Lau 2008). Speciation with gene flow may be driven by a combination of positive assortative mating and disruptive selection, particularly if selection and assortative mating act on the same trait, reducing recombination between ecotype and mating type (Udovic 1980; Dieckmann and Dobeli 1999, Fry 2003; Servidio et al 2011). Such a scenario increases the probability of speciation by eliminating recombination between the trait under divergent selection and the trait used in assortative mating (Felsenstein 1981). In chapter three we present evidence that stickleback exhibit assortative mating by diet. Among-individual diet variation leads to variation in stable isotopes, which reflect prey use. We find a significant correlation between the isotopes of males and eggs within their nests. Because egg isotopes are derived from females, this correlation reflects assortative mating between males and females by diet. In concert with disruptive selection, this assortative mating should facilitate divergence. However, the stickleback population remains phenotypically unimodal, highlighting the fact that assortative mating and disruptive selection do not guarantee evolutionary divergence and speciation.

There are several not-mutually-exclusive mechanisms by which assortative mating by diet may occur in these populations. These include direct preference for diet similarity (perhaps mediated by olfactory cues, Ward et al 2004), or indirect assortment due to shared microhabitat preference among individuals of similar diet. There are a number of prior studies suggesting that ecological difference may be associated with

nesting habitat in stickleback. In a few lakes, stickleback exist as sympatric species pairs (benthic and limnetic species), which exhibit strong ecological, morphological, and genetic differences that are sustained by assortative mating (Schluter and McPhail 1992). Benthic and limnetic stickleback differ in their nest location and characteristics, with limnetic males nesting in open, shallower areas and benthic males nesting in dense vegetation at deeper depths within the littoral zone (McPhail 1994). Females also differ in their habitat use, making encounters with males of their own species more likely for benthic and limnetic stickleback (Vamosi and Schluter 1999). Benthic-like and limnetic-like populations from allopatric solitary lakes also differ in their nest location in a manner similar to the species pairs (Vines and Schluter 2006). However, whether nesting habitat is associated with diet within variable populations of stickleback, and thereby contributes to assortative mating was unknown.

In chapter four we test whether diet-assortative mating within an ecologically variable population of threespine stickleback results from small-scale geographic isolation or microhabitat preference. We find evidence for assortative mating in the form of a positive correlation between mated pairs' diets, much like in our initial study of assortative mating by diet. Stable isotopes reveal diet differences between different nesting areas and among individuals using different nest habitat within a nesting area. Interestingly, we found the majority of associations between diet or morphology and nest habitat were opposite our *a priori* predictions from the benthic/ limnetic species pairs: males and females with stereotypically 'limnetic' traits (lower % benthic carbon, higher trophic position, Matthews et al 2010; and smaller gape width, Robinson 2000) tended to nest or lay eggs in deeper nests. Also, males guarding nests in dense vegetation had smaller gapes (a limnetic trait) and higher trophic position. The one exception was that

individuals with shorter gill raker length (a benthic trait; Robinson 2000) used deeper nests.

The observed spatial segregation of diet types should generate some assortative mating, but is insufficient to explain the observed assortment strength. Significant male-female isotope correlations remain after controlling for spatial variables. We therefore conclude that sticklebacks' diet-assortative mating arises primarily from behavioral preference rather than from spatial isolation. More generally, our results illustrate the point that spatial segregation can only drive appreciable levels of phenotypic assortative mating when environment-phenotype correlations are parallel and strong in both sexes. Consequently, intraspecific assortative mating may typically entail mating preferences rather than just spatial co-segregation of phenotypes.

An open question on the relationship between ecological divergence and magic-trait assortative mating is how assortment varies with the level of ecological divergence in a population. I have shown that ecological variation varies among populations. We also know that assortative mating likely varies among populations, with some populations showing significant assortment by diet (chapter three, chapter four) and others showing no signal of assortment (Snowberg, Stutz, and Bolnick, unpublished data). If a relationship exists between level of ecological divergence within a population and level of assortative mating this may create a feedback loop promoting further morphological and ecological divergence within populations. This in turn could change population, community, and ecosystem dynamics. The relationship between level of ecological divergence and assortative mating awaits further investigation.

Chapter 2

The magnitude of intraspecific niche variation varies across lake populations of threespine stickleback (*Gasterosteus aculeatus*)

2.1 ABSTRACT

Many ecologically generalized populations are composed of relatively specialized individuals that selectively consume a subset of their population's diet, a phenomenon known as 'individual specialization'. The Niche Variation Hypothesis (NVH) states that niche expansion during ecological release occurs mainly through diet divergence among individuals, with correspondingly higher morphological variation. We test the widely invoked but rarely evaluated assumption that diet variation within a population is correlated with morphological variation using ecologically variable lacustrine populations of three-spine stickleback. Within any given population individuals differ in microhabitat use and diet. Correlations between short and long term measures of ecological variation indicate that the degree of individual specialization varies among populations in a consistent manner over time. Finally, we find support that more ecologically variable populations are more morphologically variable. However, this only holds when total morphological variance is examined, not for individual morphological traits. This discordance may be due to different relationships between morphology and ecology between populations. We conclude that the Niche Variation hypothesis is best addressed using ecological data directly—morphological variance will only predict ecological variance in cases where the relationship between morphology and diet is quite strong.

2.1.1 Keywords:

Individual specialization, ecological variation, niche variation hypothesis, microhabitat preference, morphological variation, stable isotope analysis

2.2 INTRODUCTION

Many ecologically generalized populations are composed of relatively specialized individuals that selectively consume a subset of their population's diet, a phenomenon known as 'individual specialization' (Van Valen 1965; Roughgarden 1972; Grant and Price 1981; Smith and Skulason 1996; Wilson 1998; Bolnick et al. 2003). Individual specialization can play important roles in both evolutionary and ecological dynamics (Saloniemi 1993; Doebeli 1996; Doebeli 1997; Ackermann and Doebeli 2004; Okuyama 2008; Bolnick et al. 2011; Schreiber et al. 2011), and is also of interest to behavioral ecologists studying behavioral syndromes or consistency of behavior (Dall et al. 2012). Emerging theory on the ecological consequences of individual specialization suggests that intraspecific diet variation can stabilize population and community dynamics, promote species coexistence, and increase ecosystem productivity (Fox and Kendall 2002; Doebeli 1996, 1997; Saloniemi 1993; Bolnick et al. 2011; Schreiber et al. 2011). Some of these predicted ecological consequences have been tested experimentally, confirming that genetic variation promotes population persistence and stability and that the degree of morphological variance has strong effects on ecosystem primary productivity and prey community structure (e.g., Agashe 2009, Harmon et al. 2009, Ingram et al. 2011). Ecological variation provides the variability necessary for natural (Bolnick and Lau 2008) or sexual (Chapter 3) selection to act. Given these multifarious effects of individual specialization, it is important that biologists understand when individual specialization will be more or less pronounced (called for by Bolnick et al.

2003, reviewed in Araújo et al. 2011). If biologists can identify the biotic and abiotic factors regulating the magnitude of diet variation in populations, then it will be possible to predict when individual specialization's ecological and evolutionary consequences will be more or less important.

The Niche Variation Hypothesis (NVH) is the most prominent hypothesis as to why individual specialization may be more pronounced in some populations than in others (Van Valen 1965). The NVH was based on the observation that island populations of birds are often more morphologically variable than their mainland relatives. Van Valen (1965) suggested that release from interspecific competition led island populations to expand their niche width. Population niche expansion can arise through greater individual diet breadth, or through increased among-individual diet variation. The NVH states that niche expansion during ecological release occurs mainly through diet divergence among individuals, with correspondingly higher morphological variation (Van Valen 1965). Subsequent tests of the NVH have led to mixed support, with many studies failing to find a correlation between morphological variance and population niche width (reviewed by Bolnick et al 2007). However, these tests presuppose that morphological variance is a suitable proxy for ecological variation. Tests using direct measures of diet have generally been more supportive: populations with wider niches tend to display more individual specialization (Bolnick et al 2007 and references therein).

Why do direct ecological measurements support the niche variation hypothesis better than morphological variance? First, the NVH is fundamentally about changes in population and individual diet breadth. Following ecological release, competition within a population is expected to drive niche expansion because individuals adopting new resources can mitigate the deleterious effects of intraspecific competition. Morphology is only involved indirectly, via correlations between phenotype and resource use. Second,

even when morphology is correlated with diet, this correlation must be very strong and consistent across populations for morphological variance to be an effective proxy for ecological variance. Third, the correlation between morphological and dietary variation will be undermined if different traits affect diet in different populations. Individual specialization arises from among-individual differences in search images (arising from prior experience), foraging behavior, social status, biomechanical adaptations for locomotion, prey capture or processing, or digestive physiology. If diet depends on different combinations of morphological or behavioral traits in different populations, the morphological variance of any single trait will not be informative about diet variation. To summarize, there are multiple reasons why diet variation may be decoupled from morphological variation, which would undermine morphologically-based tests of the NVH.

Here we test the widely invoked but rarely evaluated assumption that diet variation within a population is correlated with morphological variation. We examine this relationship in the threespine stickleback (*Gasterosteus aculeatus*), a model organism for studies exploring ecological variation within and between populations. Threespine stickleback are well known for their substantial ecomorphological variation among lake, stream, and marine habitats, and between sympatric species pairs (Lavin and McPhail 1985; Schluter and McPhail 1992; Bell and Foster 1994). More recent cross-sectional studies have also found substantial among-individual diet variation within single lacustrine populations (Chapter 3; Robinson 2000; Reimchen and Nosil 2001a; Reimchen and Nosil 2001b; Reimchen et al. 2008; Bolnick and Paull 2009). On average, two individual sticklebacks from the same lake share only between 30% to 50% of their prey in common, far less than expected under a null model of random sampling from a shared

set of prey (Chapter 3; Svanbäck and Bolnick 2007; Araújo et al. 2008; Bolnick and Paull 2009; Bolnick et al. 2010).

Within populations we observe correspondingly high among-individual variance in isotopes, a measure of long term diet variability (Chapter 3; Bolnick et al 2008; Matthews et al 2010). Carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) reflect an individual's diet integrated over days to months (depending on the tissue; Post 2002; Newsome et al. 2007). Therefore, among-individual variation in isotope signatures indicates that different individuals have been consistently feeding on different types of prey (Matthews and Mazumder 2004; Araújo et al. 2007; Newsome et al. 2007). Previous studies have repeatedly detected among-individual variation in stomach contents, isotopes, and morphology, as well as correlations between these traits (Chapter 3; Svanbäck and Bolnick 2007; Araújo et al. 2008; Reimchen et al. 2008; Bolnick and Paull 2009; Bolnick et al. 2010; Matthews et al. 2010). It is also known that there is variation among populations in the amount of within-population morphological variance (Berner et al. 2010), but no similar exploration of the amount of ecological variance within populations has been carried out. Given prior evidence of individual specialization and between-population differences in morphological diversity (Matthews et al 2010; Berner et al 2010), a logical next question is to evaluate whether more morphologically variable populations exhibit stronger among-individual diet variation.

We start by evaluating the utility of cross-sectional metrics of individual specialization. We used direct observations to test whether (1) stickleback exhibit sustained among-individual differences in resource use, and (2) individuals' foraging behavior is correlated with isotopes or morphology. This represents one of only a few longitudinal diet studies of individual fish in their native habitat (Bryan and Larkin 1972; Layman et al. 2007), and the first we are aware of to use direct observations of individual

feeding behavior. Our results corroborate previous cross-sectional evidence for individual specialization in stickleback and reveal previously unrecognized dimensions of foraging variation in lacustrine fish.

We then apply cross-sectional methods to quantify individual specialization in numerous populations. Specifically we examine whether the extent of diet variation differs among populations, and whether gut content and isotopic measures of diet variation are in agreement. Finally, given evidence that individual specialization varies among populations, we test the relationship between diet and morphological variation that forms a core element of the NVH. We find support for the relationship between overall morphological variance and ecological variance among populations. We conclude by testing the primary hypothesis of the NVH that populations with broader niches have greater variation, both using morphology and diet variation. We find that morphological variation is not associated with total niche width, despite being related to ecological variation. However, similar to the results of Bolnick and colleagues (2007), we find that across populations broader population niche width is associated with higher degree of individual specialization.

2.3 MATERIALS AND METHODS

2.3.1 Enclosure observations of foraging

In the first week of June 2009, we constructed a semicircular enclosure (12 m radius; ~225 m^2) set against the shoreline of Little Mud Lake (one of the lakes in our survey, Table 1). The enclosure was built of 1/16" seine netting with lead-line and rocks sealing the lower edge to the lake bottom, and float-line and foam floats keeping the upper edge above the water surface. The enclosure location was selected to contain a

typical variety of microhabitats including flocculent benthic mud, rocks, submerged logs, emergent vegetation, and pelagic open-water ranging up to 2.5 meters deep. Note that most microhabitats were not arranged in discrete patches, but rather each was found throughout the enclosure. Thus, at any point in the enclosure a fish would be no more than about 3 meters from any of the microhabitats recorded (see below), a distance readily covered in seconds.

We chose to study Little Mud Lake because stickleback from this lake exhibit typical levels of stomach content and isotopic variance among individuals, compared with 11 other lakes in the region (Bolnick and Lau 2008, Berner et al. 2010, see survey results). As a whole, the population's diet is somewhat biased towards benthic invertebrates, but also includes zooplankton, pelagic macroinvertebrates, and aerial or terrestrial insects. Overall, Little Mud fish tend to be relatively 'benthic' in morphology, though they are still intermediate between the benthic and limnetic species pairs observed in some lakes (Berner et al. 2010). The lake harbors heritable variation for several trophic traits, which differed among lab-reared families of progeny from crosses among wild-caught Little Mud Lake parents (Bolnick unpublished results).

The enclosure was stocked with 30 stickleback, individually marked by gluing a unique color combination of small glass beads on their first dorsal spine (14/0 Rocaille seed beads: 1.0 mm wide; 0.004 g). Fish were collected immediately outside the enclosure, and a corresponding number of fish from within the enclosure were removed to maintain a natural density. Sample size was thus constrained by the area of the enclosure, and the standing density of sufficiently large fish within the enclosure.

We did not record the sex of fish prior to release, because this population is not sexually dimorphic in size, shape, or color (Bolnick and Lau 2008), so sexes cannot be reliably distinguished except by dissection. We measured each fish and used only

individuals >50mm standard length (mean SL = 62.8 mm, sd = 2.2 mm; mean mass = 3.013 g, sd = 0.434 g). Using this narrow size range makes it highly probable that all individuals are the same age, thus excluding the well-known confounding effect of ontogenetic niche shifts (Polis 1984). In addition, by using large fish, the beads comprised less than 0.2% of a fish's weight. In subsequent field observations, we noticed no qualitative effect on stickleback buoyancy or feeding behavior, when compared with unmarked fish within the same enclosure. It is possible that the added weight might somewhat shift fish towards using the benthic substrate, but should not materially influence the among-individual variation examined here.

Twenty-four hours after releasing the fish into the enclosure, a single observer (K. Hendrix) began daily snorkel observations of feeding behavior. Upon finding a marked fish, the observer followed the fish and recorded each strike on a potential prey item, noting the microhabitat where the strike took place: benthic mud, rocks, submerged logs, surface of vegetation, mid-water column, or water surface. A focal fish was followed until it was lost (visibility was ~4m), until the fish ceased to feed for at least five minutes, or for a maximum of 20 minutes. The snorkeler then moved to a different quarter of the enclosure before searching for a different fish. The first day of observation was treated as an acclimation period, and the data discarded. After the first day the stickleback exhibited no apparent response to the presence of a slow-moving snorkeler, and would feed within centimeters of the observer.

After 14 days we recaptured as many marked fish as possible, using a small aquarium net and minnow traps. Recapture rates were low, most likely due to loss of beads: a few fish lost their beads during handling on recapture, leaving us with only nine identifiable recaptures. Given this low sample size, comparisons of feeding observations with diet, morphology, and isotopes must be treated with caution. We therefore

supplement our diet, morphology, and isotope data with a dataset from 32 fish collected in the same part of the same lake in June 2006 as part of the multi-lake analysis.

2.3.2 Multi-lake survey of individual specialization

We collected 20-40 stickleback from each of 12 lakes on northern Vancouver Island, British Columbia in May and June 2006 and 2007 (Table 2.1). In addition, we collected 30 fish from a marine ecotype population in June 2009, to determine the ancestral state of diet variation for comparison with the derived freshwater populations (see appendix). Fish were captured using unbaited minnow traps placed on the benthos in a variety of microhabitats within a spatially constrained small area of each lake. Both limnetic and benthic ecotypes nest on the benthos, so this trapping strategy captures fish regardless of foraging strategy. To ensure that digestion did not obscure gut content results traps were checked at least every two hours (stickleback gut contents are significantly skewed by digestion after six hours in traps, but are reliable over shorter trap durations, Svanbäck and Bolnick unpublished results). Fish were euthanized in an overdose of MS-222 anesthetic and frozen in liquid nitrogen for transport back to Austin, TX. In addition we collected invertebrate samples (snails and mussels) representing isotopic baselines for the benthic and limnetic primary consumers (Post 2002; Matthews et al. 2010; see below) from all lakes where both invertebrates occurred.

2.3.3 Morphological, isotopic, and stomach content analysis of captured fish

In the lab, we measured several characters commonly measured in studies of stickleback feeding ecology: standard length, gape width, gill raker number and length. Gill raker length and number as well as gape width influence prey capture and handling

efficiency in stickleback (Bentzen and McPhail 1984, Lavin and McPhail 1986, Robinson 2000). On the recaptured enclosure fish we also measured some biomechanically significant traits that to our knowledge have not previously been studied in stickleback: lower jaw opening and closing lever ratios, upper jaw protrusion, and hyoid and buccal cavity lengths. Fish were sexed by inspecting gonads.

A sample of caudal peduncle muscle tissue was removed from each individual stickleback for carbon and nitrogen stable isotope analysis at the University of California at Davis Stable Isotope Facility. For most lakes we sent isotope samples for analysis from the first 30 fish with complete morphological and stomach content data. Stable isotopes are commonly used to study diet variation (Tieszen et al. 1983; Newsome et al. 2007). Carbon and nitrogen isotope ratios provide complementary information on fishes' diets. Limnetic and benthic primary producers (phytoplankton and periphyton, respectively) fix C^{12} and C^{13} isotopes in different ratios (France 1995; Post 2002). These ratios are preserved in consumers' tissues with only slight fractionation. As a result, the ratio of these isotopes ($\delta^{13}C$) provides a measure of how much benthic or limnetic carbon an individual uses (Matthews et al. 2010). Nitrogen provides a complementary measure of trophic position, because the ratio of N^{14} to N^{15} ($\delta^{15}N$) displays a stepwise enrichment at each higher trophic level (Hobson and Clark 1992a). Stable isotopes turn over slowly in tissues, integrating diet over the course of weeks to months (Hobson and Clark 1992b; Hobson 1993). Consequently, stable isotope differences among individuals may be used to infer sustained among-individual diet differences. The amount of isotopic variance a population displays can be used as a measure of long-term dietary diversity between individuals within a population, and one study has confirmed that isotopic and stomach content variation supply comparable measures of individual specialization (Araújo et. al 2007).

Using isotopic variance as a measure of population level individual specialization assumes that this variance is primarily influenced by sustained dietary variation with populations (Araújo et al. 2007). If isotopic variation reflects difference between benthic and limnetic sources rather than the amount of dietary variation within populations this metric is invalidated (Matthews and Mazumder 2004, 2005; Dalerum and Angerbjörn 2005; Araújo et al. 2007). One way to control for differences in prey isotopes is to use the methods of Post (2002) to convert $\delta^{13}C$ into a measure that reflects how far between the benthic and limnetic $\delta^{13}C$ signatures an individual is and $\delta^{15}N$ into a measure of trophic position. However, baseline samples (usually snails as primary benthic consumers and mussels as primary limnetic consumers) were not available for some of the lakes studied here (e.g., no snails could be located in three lakes, although Trichoptera were collected as a stand in for one of these lakes). Also, the conversion itself often produces results that lead to ~10% of sampled stickleback being beyond the bounds of benthic or limnetic carbon ratios, making it impossible to convert those individuals' $\delta^{15}N$ to trophic position without further assumptions. Therefore making comparisons across lakes with this procedure is questionable. We therefore test whether the difference between the benthic and limnetic baselines for $\delta^{13}C$ or $\delta^{15}N$ is correlated with the overall isotopic variability measured from the fish in that population or the var($\delta^{13}C$) or var($\delta^{15}N$), respectively. If the amount of variance in stickleback isotopes was largely due to among-population differences in how isotopically divergent the benthic and limnetic foodwebs were, rather than due to long-term differences in individual specialization, we would expect significant correlations between variance in an isotope and the baseline values across populations. All tests were non-significant ($P > 0.77$), leading us to conclude that stickleback isotopic variance reflects the amount of long-term diet variability between individuals within a population (all analyses conducted in R, R Development Core Team,

2012). We therefore conduct our analysis using the isotopic ratios of consumer stickleback and present our results with the caveat that in addition to measuring the amount of dietary variation within populations, isotopic variance may also reflect the initial baseline isotopes of the population.

Stomach contents were removed, identified to the lowest feasible taxonomic level, and counted. We calculated stomach content variation as follows. The proportional similarity (PS_{ij}) between two individuals reflects the degree to which their stomach contents contain the same prey in the same proportions (Bolnick et al. 2002). We use the standardized measure of diet variation, $E = 1 - mean(PS_{ij})$ (see Araújo et al. 2008). E theoretically varies between 0, when there is complete dietary overlap between all individuals within a population, and 1, when each individual has a completely unique diet with no shared components between individuals. However, due to a finite number of prey items present in the stomach, the actual minimum value of E is greater than zero (e.g., individuals with identical diet preferences will still have non-identical stomach contents). The degree of this bias can be estimated by a Monte Carlo resampling simulation to calculate E_{null}, the value expected if individuals sample from a shared prey distribution. We resampled the observed number of prey items for each individual from a multinomial probability distribution determined by the population average diet. This was repeated 100,000 times and at each iteration we recalculated E (using package RIS for R, Zaccarelli et al. 2011). We then rescale E to control for stochastic sampling $E_{adj} = E - E_{null}/1 - E_{null}$ which runs from 0 to 1, where 0 is the amount of diet variation expected under the null hypothesis and given the finite number of prey items observed per stomach.

To quantify the amount of variance in morphology or isotopes within a population we used the sum of eigenvalues from unscaled principal component analysis (PCA).

Morphology is measured on several different scales—to decouple trait variances and covariances from means and measurement scales we mean-scale the traits within each population separately by dividing the raw trait measurements by the corresponding population mean. This leads to a scaleless measurement with mean of 1 and variance proportional to the original variance in the population. We then did a PCA on mean-scaled mass, standard length, gape width, and gill raker length and number. The sum of the eigenvalues is equal to the sum of the variance in mean-scaled morphology. The total variance for isotopes is calculated in a similar manner but since both $\delta^{13}C$ and $\delta^{15}N$ are on the same scale (and the zero value is arbitrary) we do not mean-scale isotope values prior to our analysis.

2.3.4 Analysis: Is there individual specialization in microhabitat use within a population?

We used two methods to test for significant among-individual variation in microhabitat use in our enclosure experiment. First, a multinomial generalized linear model tested for individual and day effects on the number of strikes directed at the set of six microhabitats. An individual effect indicates that there are repeatable differences in foraging behavior among individuals. The day effect reflects between-day differences in microhabitat use for all individuals together (e.g., when a prey type emerges in large numbers). To be conservative, we restricted our analysis to individuals seen four or more occasions during the study, but equivalent results were obtained with the complete dataset.

The multinomial GLM assumes that feeding strikes are independent, which may be inappropriate if repeated unsuccessful strikes are directed at the same prey item, or if prey are spatially aggregated. A more conservative approach is to treat the observation

bout as the level of replication within a given fish. A MANOVA tested for among-individual variation in the proportional representation of the various microhabitats. We arcsine-square-root transformed the proportions prior to analysis to ensure normality of residuals. The proportion of variance explained by between-day, versus between-individual differences was estimated following Langerhans and Dewitt (2004).

2.3.5 Analysis: Is microhabitat use correlated with isotope signature and/or diet?

We examined Spearman rank correlations between individual morphological measures and microhabitat use. We used a Principal Component Analysis to summarize among-individual variation in feeding behavior (individuals were the level of replication). We retain PC axes 1 through 4, which explain more than 90% of variance in microhabitat use, for further analysis (Table 2.2). Before being compared with feeding behavior, we size-standardized gill raker length, protrusion distance, and hyoid and buccal length (residuals from regression on standard length, all variables log-transformed). Gill raker number and jaw lever ratios are independent of fish size, so were not adjusted. Given the large number statistical tests (9 traits * 4 diet axes), a correction for multiple comparisons is required. However, each test has low power individually (N = 9 fish), so that even correlations ~ 0.9 would fail a sequential Bonferroni correction. To illustrate Bonferroni's excessive conservatism, six out of nine tests for trait associations with feeding PC3 yield $P < 0.1$ (Table 2.3), which is highly improbable under a null model. We therefore adjusted for multiple comparisons not by Bonferroni correction to individual p-values, but by a Fisher's combined probability test evaluating whether the suite of characters have a jointly significant correlation with feeding behavior.

Following Post (2002), we calculated the percentage of benthic carbon in each individual's diet, using mussel and snail isotopes as a benchmark. In the larger (N = 30) sample from 2006 we used a linear model to test for a correlation between individuals' isotope signature ($\delta^{13}C$ or $\delta^{15}N$), sex, and morphology (standard length, raker number, size-adjusted raker length, size-adjusted gape width). Within the smaller sample (N = 9) for which we had both isotope ratios and behavioral observations, we tested for a significant Spearman rank correlation (one tailed test) between the proportion of benthic carbon in an individual's muscle tissue, and the proportion of feeding strikes directed at benthic surfaces.

2.3.6 Analysis: Variation in individual specialization and morphology across populations

We tested whether there is significant individual specialization in all studied populations by comparing the observed value of E to the distribution of the simulated values calculated through our Monte Carlo resampling of E_{null}. We then rescaled E to E_{adj}, as described above, and tested whether this gut content based metric of individual specialization is correlated with the amount of isotopic variation among populations. Such a correlation would support individual specialization being a long-lasting phenomenon in populations.

Whether morphological variance should predict ecological variance depends on how strong and consistent relationships between morphology and diet are within and among populations. We tested whether the relationship between morphology and isotopes differs between populations for each isotope. We used stepwise model selection (using the R package MASS, Venables and Ripley 2002) to choose the best fit model starting with individual morphology and potential sex and lake (population) interactions with

morphology. We focused on both how morphology predicts long-term diet in similar manners across populations (without interactions with lake) and significant interactions between morphology and population showing inconsistencies in how morphology predicts diet across populations.

To test whether morphological variance is associated with ecological variance we tested for correlations between isotopic variance or E_{adj} and morphological variance. Because a positive correlation between each type of variance is the clear *a priori* prediction we report one-tailed statistics for these tests. In addition we tested whether variance in any single morphological trait is correlated with variance in isotopes or diet (E_{adj}).

The NVH predicts that populations with greater total niche width will have higher degree of morphological variation. While this has received limited support it has been suggested that a more clear relationship between ecological release and individual specialization may exist (Bolnick et al 2007). We therefore follow Bolnick et al. (2007), which tests for a correlation between total niche width (TNW) and a different metric of individual specialization, V, in an experimental manipulation of intraspecific competition within one population of stickleback. We calculated TNW using discrete prey counts using the Shannon-Weaver diversity index (Roughgarden 1972, 1979). To stay in line with the methods of Bolnick et al. (2007) we used lumped functional categories for prey in these analyses, although interpretation of our results remains the same if unlumped prey counts are used. V is equal to 1-mean(PS_i), which is intuitively very similar to E, with the primary difference being that rather than comparing the diets of each pair of individuals, PS_i compares the diet of each individual with the population mean diet. Much like E, higher values of V represent greater individual specialization. We tested for a correlation between TNW and V to determine whether populations with greater niche

width show greater ecological variance. We also tested for a correlation between TNW and total morphological variance.

2.4 RESULTS

2.4.1 Observations of foraging behavior

We observed 25 of the 30 marked fish during the 14-day enclosure study. Only one carcass of a marked fish was observed, suggesting that unobserved individuals either lost their beads, were eaten by predators (trout were occasionally observed in the enclosure), or escaped over the top edge of the enclosure. No marked fish were observed outside the enclosure.

The mean duration of individual observations was 10 minutes, and included a median of 19 distinct prey strikes (ranging from 1 to 85, 1st and 3rd quartiles: 9 and 33). On average individuals were observed on 5.4 occasions (sd = 2.4) with a maximum of 11 periods of observation. Across all days, individuals were seen to execute as few as 7 strikes at prey (an individual observed only once), up to 297 strikes (mean = 126 strikes, sd = 89). While we did not systematically collect data on movement distances during observation periods (movement was rarely linear), several fish were seen to move throughout the entire enclosure during a single observation period. Movement distances were usually at least five meters, with the result that all individuals had ready access to all possible microhabitats.

2.4.2 Microhabitat individual specialization

Individual stickleback typically foraged in a mixture of microhabitats both within and across days. We observed fair repeatability of individuals' microhabitat use across days. For instance, one individual focused its attacks almost exclusively benthic mud (~88%) on each of eight observation days, whereas another individual rarely exceeded 20% benthic strikes over seven observations (typically 0%). A minority of individuals were observed to use microhabitats more evenly, or switched habitats across days such as one individual that foraged on 85% benthic mud one day, 100% mid-water strikes another, and 90% surface strikes on a third day.

Despite some day to day variation in individual diets, stickleback exhibited substantial among-individual variation in microhabitat use (Figure 2.1). On average, pairs of individuals share only 44% of their microhabitat use (mean $PS_{ij} = 0.44$; $E_{adj} = 0.426$). Focusing on individuals observed for four or more days, a multinomial GLM found significant support for among-individual variation in foraging behavior (LR = 1347.24; $P << 0.0001$). There was also an observation day effect (LR = 187.87; $P < 0.0001$) indicating that on certain days all fish shifted towards a particular microhabitat, although between-individual variation effect size was much greater. A more conservative test treating observation-period as the level of replication also provides overwhelming support for among-individual variation (MANOVA: Wilk's $\lambda = 0.131$, df = 16, $P < 0.0001$), but found no significant day effect (Wilk's $\lambda = 0.587$, df = 7, P = 0.159). Individual identity explained 29% of the variation in microhabitat use among observations, compared with 8% attributed to between-day variation across all individuals. Analyses of all individuals (as opposed to those with ≥ 4 observations) yielded similar inferences and levels of support.

Foraging behavior variation did not simply fall on a single benthic/limnetic axis (Table 2.2). Principal component analysis found the first four axes collectively explain 92% of the cumulative variance in proportional use of the six microhabitats. The first major axis was indeed a benthic versus limnetic (mid-water) axis, but explained only 40% of the variance among individuals. Two orthogonal axes (PC2&3) distinguished individuals that attacked the water surface versus non-surface sites (Table 2.2). PC4 distinguished individuals that fed on different types of hard substrate (logs vs. vegetation and rocks).

2.4.3 Comparing among-individual variation in diet, isotopes, and microhabitat

Stomach contents revealed significant among-individual variation in prey use (2006 E_{adj} = 0.526; 2009 E_{adj} = 0.463, P< 0.0001). Both values are marginally larger than the amount of among-individual variation in microhabitat use (E_{adj} = 0.426). Stable isotopes of muscle tissue provide additional evidence for sustained diet variation among individuals, both in 2006 and in the 2009 enclosure (Figure 2.2). Focusing on 2006 collections, each isotope ratio exhibited substantial variation (sd(δ^{13}C) = 1.47; sd(δ^{15}N) = 0.66). For comparison, laboratory-reared fish fed a shared diet of chironomids exhibited substantially less isotopic variation (sd(δ^{13}C) = 0.660; sd(δ^{15}N) = 0.228; Chapter 3). Using mussels and snails to designate ends of a limnetic-benthic continuum, we estimate that individuals ranged from 10% to as high as 100% benthic carbon (mean = 66.1, sd = 25.0). Relative trophic position (a linear transformation of δ^{15}N) is correlated with this index (2006: r = -0.83, P < 0.001; 2009: r = -0.86, P = 0.003). This correlation is typical of stickleback populations in many lakes, most likely because pelagic fish consume a higher proportion of predatory zooplankton as opposed to the primary consumers eaten

by benthic stickleback (Matthews et al. 2010). There was a marginally significant positive relationship between the proportion of strikes directed toward the benthos and the percent benthic carbon isotopes for recaptured individuals (Figure 2.3, rho = 0.58, *P* = 0.054).

2.4.4 Causes of among-individual habitat use variation: sex, size, morphology

Among-individual variation in foraging microhabitat may reflect differences in sex, age, size, morphology, and/or behavior. We found no significant effect of sex on relative use of microhabitats (MANOVA, P = 0.280). Analysis of stomach contents also revealed no significant difference among sexes (MANOVA; 2006, P = 0.07; 2009, P = 0.33). Stable isotopes also did not differ between sexes in either year (P > 0.3 for $\delta^{13}C$ and $\delta^{15}N$ in each year).

Ontogenetic niche shifts do not contribute to the present study, as all individuals were adults and likely members of a single cohort (1 year old). However, size may vary within a cohort. Using the standard lengths measured before releasing the enclosure fish, we found a marginally significant correlation with feeding PC1 (P = 0.09), but no other axis of feeding variation (Table 2.2). The recaptured fish were measured more precisely with digital calipers, and using this smaller sample we found a significant trend for longer individuals to feed more in mid-water as opposed to benthic mud (feeding PC1, P = 0.014), but no effect on other feeding axes (Table 2.3).

Despite the low sample size of recaptured fish, we also found significant correlations between feeding behavior and several morphological traits (Table 2.3). Fish feeding more on the water surface (as opposed to vegetation and logs [PC2] or benthos or mid-water [PC3]) tended to have longer gill rakers (adjusted for body size), narrow

gapes, low opening lever ratio of the lower jaw, and higher size-adjusted buccal length. Fish feeding on logs (as opposed to vegetation or rocks, PC4) had longer size-adjusted hyoid length. Although no individual correlation survived sequential Bonferroni corrections, more tests were significant ($P < 0.05$) or approached significance ($0.1 > P > 0.05$) than could be explained by chance (Fisher's combined probability $P = 0.0005$ using all 36 tests; Table 2.3). In particular, feeding PC2 and PC3 exhibit consistently low P-values across multiple tests (Table 2.3). We can therefore confidently conclude that foraging variation is correlated with morphological differences among individuals, but treat any particular trait/behavior correlation with caution due to our low recapture sample size.

2.4.5 Individual specialization varies among populations

All populations sampled, including the marine ecotype population, showed significant individual specialization (the value of E was greater than expected under Monte Carlo simulation of E_{null}, $P < 0.00001$ for all populations). The magnitude of diet variation differed among populations, judging by variation in both E_{adj} and the amount of isotopic variance (E_{adj} ranges from 0.383 to 0.657, isotopic variance ranges from 1.192 to 3.906, Table 2.4). Importantly, the amount of individual specialization measured through stomach contents (E_{adj}) is significantly correlated with the amount of total isotopic variance in a population ($r= 0.674$, $P= 0.008$; Figure 2.4, Table 2.4). Agreement between these independent metrics reinforces the observation that populations exhibit different levels of diet variation. This relationship also holds when comparing E_{adj} and carbon isotope variance ($r= 0.640$, $P= 0.014$), but not nitrogen isotope variance ($r= 0.251$, $P= 0.21$). These results also hold if E_{adj} is calculated by first combining prey types into

functional categories such as benthic cladocerans, limnetic cladocerans, benthic copepods, etc.

2.4.6 Comparing trait-diet correlations among populations

Trait-diet relationships differ among different populations. $\delta^{13}C$ was correlated with size-adjusted gill raker length in the entire dataset, but the slope of this relationship varied among populations (lake*raker length interaction; Figure 2.5). $\delta^{13}C$ also depended on sex, a significant interaction between gill raker number and population, and a three way interaction between gill raker length, sex, and population. $\delta^{15}N$ also differed among populations and between sexes and had a global relationship with standard length and residual gill raker length. There were significant interaction terms between sex and population, gill raker number and population, standard length and sex, and a three-way interaction between standard length, sex, and population (Table 2.5B). In conclusion, the relationship between diet and particular morphological traits varied among populations and between males and females. Thus, a multi-trait measure of morphological diversity might be a better guide to predicting dietary diversity than is the variance of any single morphological trait.

2.4.7 Comparing diet and morphological variances

The amount of isotopic variance in each population is correlated with the amount of morphological variance in each population (r= 0.538, P= 0.036; Figure 2.6, Table 2.4). Despite this overall correlation there are no significant correlations between variance in single morphological traits and overall isotopic variance or variance in single isotopes (all

$P > 0.15$), as might be expected from the varying relationships between diet and morphology found above.

2.4.8 Testing the niche variation hypothesis

TNW was correlated with individual specialization measured from gut contents, V ($r= 0.6179$, $P= 0.024$) as has previously been found for a single population of stickleback under different levels of intaspecific competition (Bolnick et al 2007). However, morphological variance was unrelated to total niche width ($r= 0.2584$, $P= 0.42$).

2.5 DISCUSSION

Our results corroborate key elements of the Niche Variation Hypothesis (Van Valen 1965). Within any given population individuals differ in microhabitat use and diet. Direct observation of individual foraging microhabitat use in one population showed that that individual stickleback consistently differed in their microhabitat use and that microhabitat use is correlated with individual long-term prey usage (measured with stable isotopes; Figure 2.3) and functional morphology (Table 2.3). This result corroborated the use of stomach contents and stable isotopes in measuring individual specialization. We then applied these tools to measure individual specialization in multiple lacustrine populations of stickleback to determine how and why ecological variation itself varies between populations. All populations, including an ancestral-type marine ecotype population, display individual specialization, but correlations between stomach-content and isotope measures of variance indicate that the degree of individual specialization varies among populations. Finally, we find support that more ecologically variable populations are more morphologically variable. However, this only holds when total

morphological variance is examined, not for individual morphological traits. This discordance may be due to different relationships between morphology and ecology between populations or the relationships between diet and morphology not being strong enough to be detected when using univariate morphological traits to test whether variance in morphology is related to variance in ecology. We conclude that the NVH is best addressed using ecological data directly—morphological variance will only predict ecological variance in cases where the relationship between morphology and diet is quite strong.

2.5.1 Microhabitat use and individual specialization

In focal follows of individual stickleback in the wild, each individual preferentially used one of the available microhabitats more often than the others, though there is across-day variation in each fish's relative use of the six microhabitats. Importantly, individuals varied widely in their propensity to attack prey on benthic substrates, mid-water column, the water surface, or on the surfaces of submerged vegetation, rocks, or logs. On average, a pair of fish from our study were about 43% divergent in their relative use of alternative microhabitats (E_{adj} = 0.426). For comparison, paired individuals were on average about 46% divergent in their relative use of prey taxa (based on stomach contents, E_{adj} = 0.463). Thus, variance in microhabitat use may explain a significant portion of the variance in prey use among co-occurring individuals. Diet variation could exceed microhabitat-use variation if individuals partition prey even within a microhabitat, as has been shown experimentally in stickleback (Araújo et al. 2008).

The variation in feeding location took place inside a constrained area of a lake, thus minimizing the effect of spatial heterogeneity. For example, due to the depth of our enclosure (2.5 m maximum), the three major categories of microhabitat use (benthic mud, mid-water, and surface feeding) were always immediately adjacent. An individual could in principle switch from surface to mid-water feeding, or benthic to mid-water feeding, by moving mere centimeters, and even benthic versus surface foraging sites were typically only a meter or two apart. Fish typically swam further than this over the course of each observation bout. Consequently, we conclude that feeding variation represents individuals' preferences rather than an effect of coarse-grained spatial heterogeneity that limits individuals' access to various feeding sites. This is consistent with previous findings that diet variation is maintained even in small (10 m^2) enclosures that ensure all individuals have equal access to all prey (Svanbäck and Bolnick 2007; Bolnick et al. 2010) .

A secondary question concerns the mechanisms that might underlie this among-individual variation. We find no evidence for between-sex diet variation, contrary to some previous studies (Reimchen and Nosil 2001a; Reimchen and Nosil 2004; Spoljaric and Reimchen 2008), though our sample sizes are too low reject very weak sex effects. Larger sample size studies of stable isotopes (N > 300) reveal between-sex differences in diet in some lakes (e.g., Cedar Lake; Bolnick et al, manuscript) but not others (e.g., Roberts Lake; Bolnick and Paull 2009) within the same watershed. We can also rule out ontogenetic niche shifts because the stickleback in this study were all large adults and likely members of a single cohort.

Evidence for correlations between size and microhabitat use are ambiguous, and depend on whether we use the entire set of feeding observations or just the recaptured fish. Although standard length of the recaptured fish yields the strongest correlation with

feeding behavior (Table 2.3), this axis of behavior (PC1) fails the Fisher's combined probability test so we cannot rule out the possibility that its correlation with standard length is a false positive. Finally, analysis of the 2006 sample revealed no correlation between body size and stable isotope-based measures of diet, or stomach contents.

In contrast, gill raker length and gape width are each correlated with two feeding behavior axes (PC2 and PC3) that are clearly related to some morphological characters (Fisher's test, Table 2.3). These results are consistent with numerous prior studies of stickleback individual specialization (Chapter 3; Schluter and McPhail 1992; Robinson 2000; Bolnick 2004a; Bolnick et al. 2008; Spoljaric and Reimchen 2008; Bolnick and Paull 2009; Matthews et al. 2010), though ours is the first to relate morphology to microhabitat use in the field. In addition to commonly-studied traits, we examined several biomechanical traits that have not previously been examined in relation to stickleback foraging ecology. We found an intriguing but not quite significant tendency for opening lever ratios to be negatively correlated with feeding on the water surface (both PC2 and PC3, Table 2.3). The lever ratio represents mechanical efficiency of force and velocity during jaw movement. Higher lever ratios result in jaw movements that directly translate more of the input muscle force to the jaw tip than lower ratios (Westneat 1994). Thus, fish feeding on less elusive benthic prey may be adapted to produce powerful but slower jaw movements (Wainwright et al. 2004). Surface-feeding fish also tended to have higher buccal length (capable of engulfing relatively more water during a feeding event). The ability to entrain a greater volume of water is expected to be beneficial when pinpointing the location of a prey item is difficult, such as when an individual is feeding near the air-water interface. While not conclusive at present, the lever ratio and buccal length results suggest that more biomechanically sophisticated studies of stickleback may improve our understanding of the morphological basis of diet variation in stickleback.

It is important to note that the trait-diet correlations described above could arise because morphology dictates feeding behavior, and/or because feeding behavior alters morphology via phenotypic plasticity (Meyer 1987; Day et al. 1994; Svanbäck and Eklöv 2002; Sharpe et al. 2008). It is likely that some diet variation reflects among-individual behavioral differences arising from previous experience (e.g., search image, Persson 1985), or heritable behavioral traits (e.g., risk-aversion, Bell 2005). Purely behavioral differences among individuals could ultimately alter morphology via diet-induced phenotypic plasticity. However, several lines of evidence suggest that morphology underlies diet variation in this study. First, gill raker and gape traits alter prey-capture efficiency in lab-reared stickleback (Robinson 2000), and prey preferences in wild fish (Araújo et al. 2008). Second, these traits exhibit heritable variation in Little Mud Lake (Bolnick, unpublished results). Finally, previous competition experiments have found that prey-switching is conditional on individuals' morphology.

In conclusion, we show for the first time that stickleback exhibit persistent among-individual variation in microhabitat use while foraging. Because prey taxa differ between these microhabitats, variation in feeding site translates into appreciable differences in diet between individuals. As a result, individuals use a subset of the population's resource base. Our results also corroborate the role of morphology in diet variation, and suggest that some additional biomechanical traits should be examined more closely in future studies of stickleback feeding biology.

2.5.2 Variation in individual specialization across populations

All populations in our survey, including the ancestral-type marine ecotype, showed significant individual specialization. Strength of interpretation is gained by

having multiple lines of evidence for ecological variation. Specialization varied between populations—snap-shot gut content metrics and longer-term isotope based metrics of dietary variation were correlated among populations (Figure 2.4). This correlation suggests the degree of individual specialization observed using stomach contents persists for at least the duration of time measured by isotopes (one to several months). Consequently, we infer that there are sustained among-population differences populations in the degree of individual specialization.

Another prediction of variation in individual specialization is that we expect populations which are more morphologically variable to have a greater degree of individual specialization. Following this expectation we find populations that have a greater degree of morphological variation also display greater individual specialization measured by isotopic variance (Figure 2.6). While this result has been theoretically predicted, it has not previously been demonstrated that the amount of individual specialization varies across populations in a predictable manner related to variation in functional morphology. Greater morphological variation tends to be associated with intermediate lake size (Bolnick & Lau 2008; Berner et al. 2010). We therefore would expect higher levels of individual specialization in intermediate size lakes.

Despite the relationship between overall morphological variance and isotopic variance we fail to find any correlations between variance in single morphological traits and any measure of individual specialization (overall isotopic variance, variance in individual isotopes, or E_{adj}). While there are global relationships between isotopes of individuals and morphological traits (for example gill raker length residuals and $\delta^{13}C$ isotope ratios, Table 2.5A), there are also differences in the relationships between these traits and isotopes between populations as indicated by significant lake interaction terms. Variation between populations in how morphology and diet are linked is one potential

explanation as to why variation in single morphological traits fails to predict population level ecological variability. This suggests that most of the previous work on ecomorphology in fish has oversimplified by assuming that diet-trait correlations observed in one population can be extrapolated to other populations as well. Further work is required to determine why diet-trait correlations differ among populations.

2.5.3 Implications

Very little is known about how the degree of individual specialization might alter the dynamics of ecological communities. A number of theoretical models suggest that intraspecific diet variation may profoundly alter the dynamics of single populations (Fox and Kendall 2002) or predator-prey interactions (Doebeli 1996; Doebeli 1997; Savolainen and Vepsalainen 2003; Schreiber et al. 2011). Direct test of these predicted ecological consequences have begun, confirming that genetic variation promotes population persistence and stability and that the degree of morphological variance has strong effects on ecosystem primary productivity and prey community structure (e.g., Agashe 2009, Harmon et al. 2009, Ingram et al. 2011). Documenting empirical patterns of individual specialization, as we have done here, is a necessary first step towards addressing broader questions about the consequences of niche variation within natural populations. Ecologically variable populations of stickleback may make a good model for understanding the effects of variation in specialization on population, community, and ecosystem dynamics.

2.6 SUPPLEMENTARY MATERIAL

2.6.1 Analysis: Comparing individual specialization in lake and marine ecotype populations

Freshwater stickleback on Vancouver Island were derived from ancestral marine population after the retreat of glaciers approximately 12,000 years ago (Clague and James 2002). These marine ecotypes are believed to have undergone very little genetic or morphological change since the founding of freshwater populations (Bell and Foster 1994) and therefore represent a good estimate of the ancestral state of morphological and ecological variance that is currently observed in freshwater populations. However, there is no available data on diet or individual specialization in marine populations.

We collected a sample of marine ecotype three-spine stickleback from a brackish population in Salmon River Estuary (Table 2.1) to compare morphological and ecological variability between derived lake and marine ecotype populations. We did not use this sample in our analysis of the relationships between different measures of ecological variation but instead compare the level of individual specialization and isotopic and morphological variation calculated from this marine population with the sample of lake populations using one-sample t-tests. We do not have *a priori* predictions as to whether ecological specialization and population variation should be larger or smaller in the marine population than in lake populations so we present our results with two-tailed *P*-values. In addition to metrics of variation already discussed we compare prey richness (the number of types of prey found in each population's diet) between the marine and freshwater populations.

2.6.2 Results/Discussion: Comparing individual specialization in lake and marine ecotype populations

The level of individual specialization observed in gut contents in the sample marine ecotype Salmon River estuary population was lower than the mean of E_{adj} in lake populations (one-sample t-test, t = 4.9763, d.f. = 11, P = 0.00042, Table 2.4). This may partially represent a lower diversity of prey available in brackish environments as most aquatic insects, which make up an important component of lacustrine stickleback diets, require fresh water environments. Prey richness (number of prey categories seen in the diet) and total niche width were also smaller for the marine ecotype population than derived freshwater populations (Table 2.4). In addition to differences in prey richness, the majority of the prey categories found in the marine population were not well represented in the overall marine diet. Total niche width (measured though a Shannon-Weaver diversity index, Roughgarden 1979) is increased by both an increase in prey richness and evenness of different categories.

Similar to the results based on stomach contents the amount of isotopic variability was marginally lower in the estuary population (t = 2.1743, d.f. = 11, P = 0.052). Variance in $\delta^{13}C$ (the generally more variable isotope) was lower in the estuary population (t = 2.5788, d.f. = 11, P = 0.026) whereas variance in $\delta^{15}N$ was higher in the estuary population (t = -10.0153, d.f. = 11, $P < 0.0001$). We do not have additional isotope data to represent benthic and limnetic primary consumers for the Salmon River estuary population, limiting our ability to determine whether the decrease in $\delta^{13}C$ and increase in $\delta^{15}N$ variance is due to differences in environmental isotopes or differences in prey consumption patterns, but the lower level of individual specialization measured through gut contents does correspond with the lower overall level of isotopic variance

observed. Thus, while individual specialization was present in the marine ecotype population, it was found to be less pronounced than in derived freshwater populations. Despite lower individual specialization in the marine population, there was no difference or greater morphological variance in the estuary population. Two morphological characters (gill raker number: t = -3.2085, d.f. = 11, $P = 0.0083$; residual gape width: t= -7.0913, d.f. = 11, $P < 0.0001$) showed increased variance in the marine population. Overall morphological variability, as well as variance in other morphological traits measured, did not differ between the lake samples and Salmon River estuary (overall: t = -0.5848, d.f. = 11, $P = 0.57$; standard length: t = -1.2787, d.f. = 11, $P = 0.23$; residual gill raker length: t = -0.6006, d.f. = 11, $P = 0.56$). Curiously, these results differ from those of Berner et al (2010), who found overall higher phenotypic variance but strikingly lower variance in gill raker length in the marine population compared to freshwater. The disagreement between our results and Berner et al is particularly striking given that we sampled the same marine population and many of the same freshwater lakes, suggesting that phenotypic variance might fluctuate over time within populations. This possibility bears further investigation.

2.7 ACKNOWLEDGEMENTS

We thank Tania Tasneem and On Lee Lau for help collecting survey fish. Chris Harrison and Bob Rodbumrung assisted with the setup of the microhabitat enclosure. Kim Hendrix collected behavioral and morphological data for the cross-sectional specialization study. Rose Carlson assisted with functional morphology measurements on recaptured enclosure fish. This work was funded by NSF DEB-0412808 and a David and Lucille Packard Foundation fellowship to DIB. LKS was supported by a NSF GRF.

Population	Latitude (N)	Longitude (W)	Year	# Fish	# Isotopes
Cecil Lake	50°14’13”	125°32’35”	2006	35	30
Dugout Lake	50°10’57”	125°31’26”	2006	20	20
Gosling Lake	50°02’43”	125°30’41”	2006	35	30
Gray Lake	50°03’27”	125°35’40”	2006	35	30
Little Goose Lake	50°09’49”	125°29’17”	2006	30	20
Little Mud Lake	50°12’23”	125°33’00”	2006	32	30
McCreight Lake	50°17’08”	125°38’46”	2007	33	30
McNair Lake	50°13’40”	125°34’31”	2006	32	30
Mud Lake	50°12’01”	125°33’59”	2006	30	30
Ormund Lake	50°10’49”	125°31’30”	2006	40	40
Roberts Lake	50°12’45”	125°32’03”	2006	27	27
Second Lake	50°03’28”	125°47’03”	2006	30	30
Salmon River Estuary	50°22’38”	125°57’05”	2009	30	30

Table 2.1: Collection information for survey study.

	PC1 Loadings*	PC2 Loadings*	PC3 Loadings*	PC4 Loadings*
Benthos	-0.62	0.00	-0.14	-0.21
Mid-water	0.56	0.01	-0.19	-0.14
Surface	0.38	0.43	0.52	0.17
Rock	0.28	-0.17	-0.71	0.36
Vegetation	0.27	-0.57	0.37	0.58
Logs	0.27	-0.57	0.16	-0.67
% variance	40.1	20.9	19	12.3

Table 2.2: Principal component analysis of among-individual variation in proportional use of various microhabitats while foraging.

* Loadings represent the strength of association between each microhabitat and each PC axis, in an overall PCA with all individuals. PC5 and PC6 explain little variance, have no significant elements, and so are not presented.

	Microhabitat PC1†		**Microhabitat PC2**		**Microhabitat PC3**		**Microhabitat PC4**	
	ρ	***P***	**P**	***P***	**ρ**	***P***	**ρ**	***P***
Standard length	**0.80** ‡	**0.014**	0.33	0.385	0.07	0.880	0.20	0.613
Gape width *	-0.43	0.250	*-0.63*	*0.070*	**-0.70**	**0.043**	-0.57	0.121
Gill raker number	0.00	1.000	0.17	0.671	*0.64*	*0.065*	0.25	0.512
Gill raker length *	0.26	0.493	**0.7**	**0.043**	**0.73**	**0.022**	0.5	0.178
Closing lever ratio	-0.05	0.912	0.15	0.708	-0.33	0.385	-0.08	0.843
Opening lever ratio	-0.37	0.330	*-0.64*	*0.065*	*-0.64*	*0.065*	-0.51	0.160
Protrusion distance *	-0.03	0.948	0.50	0.178	-0.38	0.313	-0.07	0.880
Hyoid length *	0.38	0.312	0.58	0.108	0.67	*0.058*	**0.70**	**0.043**
Buccal length *	0.35	0.359	*0.63*	*0.076*	*0.67*	*0.058*	0.47	0.213
Fisher's χ^2		19.6		33.5		*40.7*		23.6
Combined P		0.355		*0.014*		*0.002*		0.167

Table 2.3: Spearman rank correlations (ρ) and their P-values for associations between morphological traits and foraging behavior, for 10 recaptured individuals.

* Size-adjusted by obtaining residuals of log-transformed trait values against log-transformed standard length.
† See Table 2.2 for PC axis loadings.
‡ Traits with P-values less than 0.05 are in bold, less than 0.10 are italicized.

Lake	E_{adj}	Richness	TNW	Morph Var	Iso Var
Cecil	0.657	18	1.966	0.190	3.078
Dugout	0.509	17	1.906	0.246	1.818
Gosling	0.605	24	2.240	0.409	6.025
Gray	0.548	22	2.649	0.273	2.290
LM	0.525	17	1.844	0.444	2.613
McCreight	0.614	20	2.331	0.290	2.932
McNair	0.402	21	1.555	0.133	2.259
Mohun	0.630	16	2.178	0.504	4.620
Mud	0.647	19	1.816	0.284	3.934
Ormund	0.383	20	2.526	0.247	1.192
Roberts	0.589	25	1.638	0.230	3.906
Second	0.449	19	2.128	0.264	2.738
Salmon River Estuary	0.410	16	1.657	0.311	2.287
t value	5.01	4.76	4.18	-0.58	2.17
P	0.0004	0.0006	0.0015	0.57	0.052

Table 2.4: Observed values of measures of individual specialization, niche width, and total morphological and isotopic variance for 12 lacustrine populations and one marine ecotype population (Salmon River Estuary). t and *P* values compare the values for the marine ecotype to those of the lacustrine populations using a one-sample t-test.

A. $\delta^{13}C$ Isotope Ratios

	Sum Sq	Df	F value	*P*
Lake	387.89	11	18.77	< 0.0001
Sex	28.92	2	7.70	0.00056
Standard Length	5.35	1	2.85	0.093
Gill Raker Number	0.01	1	0.0008	0.98
Gill Raker Length*	58.77	1	31.28	< 0.0001
Lake x Sex	38.91	12	1.73	0.061
Lake x Standard Length	35.94	11	1.74	0.065
Lake x Gill Raker Number	46.79	11	2.26	0.012
Lake x Gill Raker Length*	40.89	11	1.98	0.031
Sex x Standard Length	5.44	2	1.45	0.24
Sex x Gill Raker Length*	0.80	1	0.43	0.51
Lake x Sex x Gill Raker Length*	40.24	11	1.95	0.034
Residuals	509.10	271		

B. $\delta^{15}N$ Isotope Ratios

	Sum Sq	Df	F value	*P*
Lake	114.538	11	38.22	< 0.0001
Sex	3.547	2	6.51	0.0017
Standard Length	1.714	1	6.29	0.013
Gill Raker Number	0.174	1	0.64	0.42
Gill Raker Length*	3.568	1	13.10	0.00035
Lake x Sex	9.291	12	2.84	0.0011
Lake x Standard Length	4.850	11	1.62	0.093
Lake x Gill Raker Number	6.298	11	2.10	0.020
Sex x Standard Length	2.217	2	4.07	0.018
Lake x Sex x Standard Length	8.363	11	2.79	0.0018
Residuals	77.100	283		

Table 2.5: Best fit model results for $\delta^{13}C$ (A) and $\delta^{15}N$ (B) isotope ratios. Models started with fish morphology (standard length, gill raker number, and residual of gape width and gill raker length) with potential two-way and three-way interactions with sex and lake. All factors from the best fit model are included.

* Size-adjusted by obtaining residuals of log-transformed trait values against log-transformed standard length.

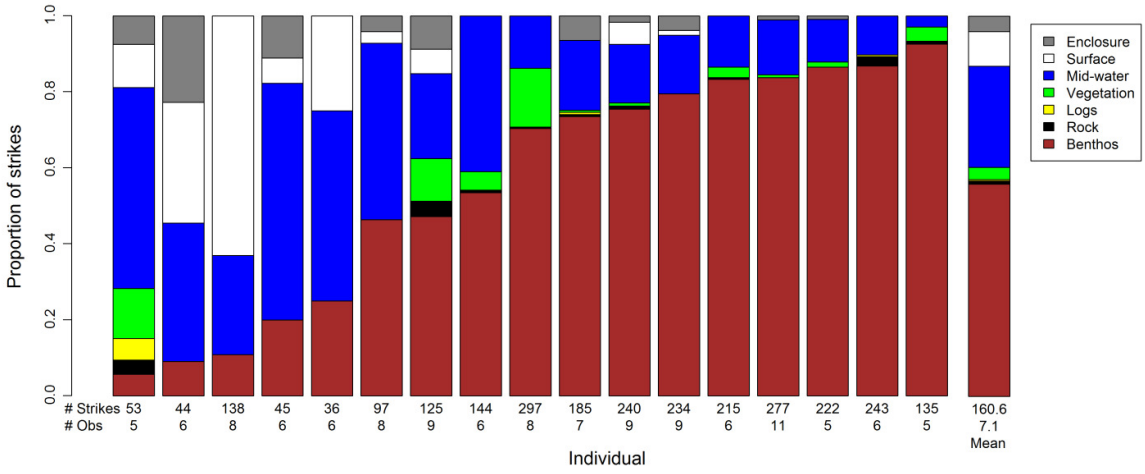

Figure 2.1: Foraging behavior variation among individual stickleback. Each individual is represented by a vertical bar, subdivided to represent the proportion of feeding strikes that the individual directed against various microhabitats (designated by colored shading). Numbering below each bar presents the total number of feeding strikes observed for the individual (first number), and the number of times the individual was observed (second number). We only present those individuals that were observed on at least four separate occasions. The bar on the right represents the mean habitat use by the individuals represented in this figure, which was very similar to the overall mean habitat use by all individuals.

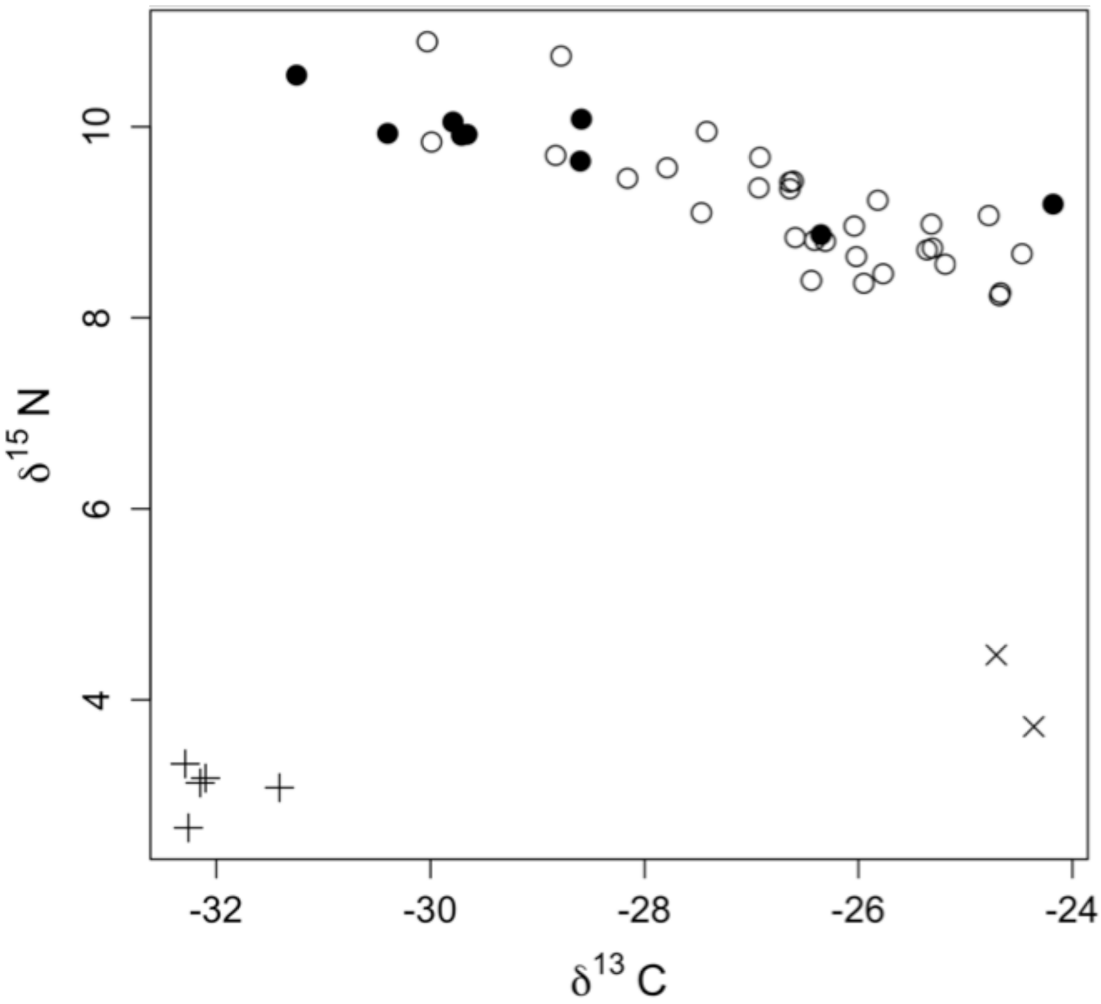

Figure 2.2: Carbon and nitrogen stable isotope signatures (δ^{13}C and δ^{15}N) of recaptured fish (solid dots), wild-caught fish (2006 sample, open dots), pelagic primary consumers (mussels; +) and benthic primary consumers (snails; ×).

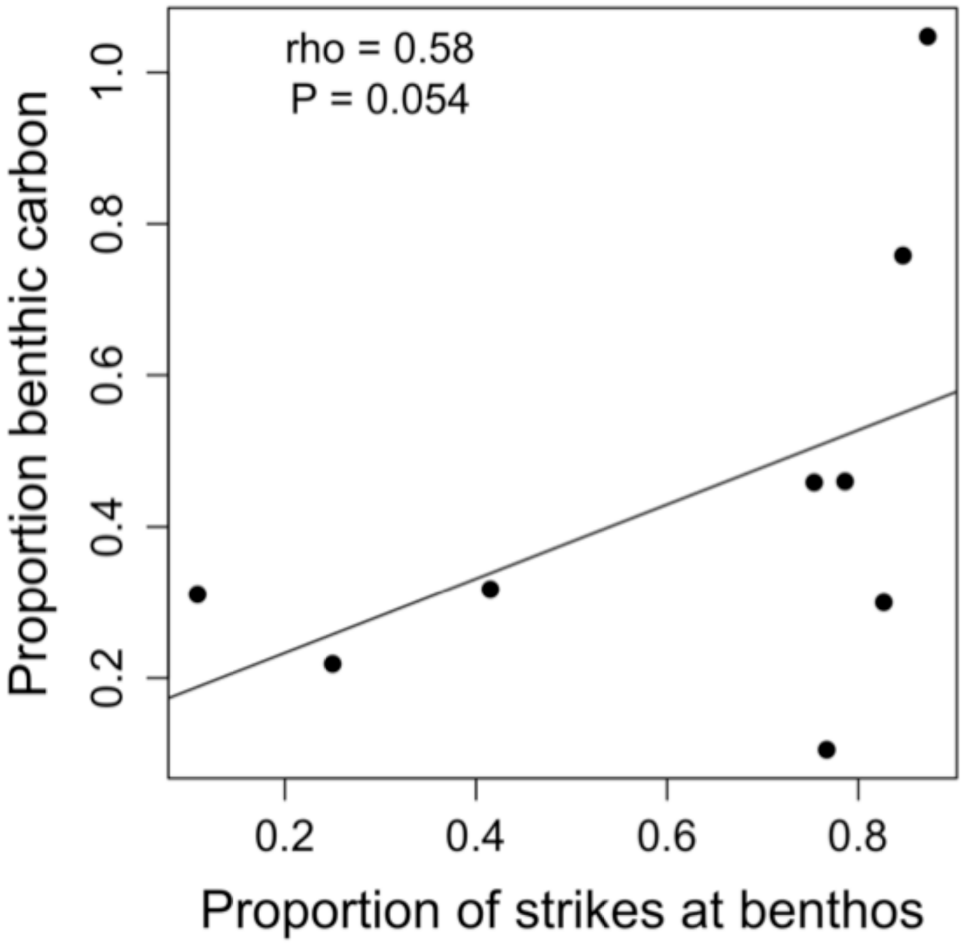

Figure 2.3: A comparison of individuals' isotopic signature (estimated proportion benthic carbon) and their observed feeding behavior (proportion of strikes on benthic surfaces). Spearman rank correlation (rho) and P-values are provided.

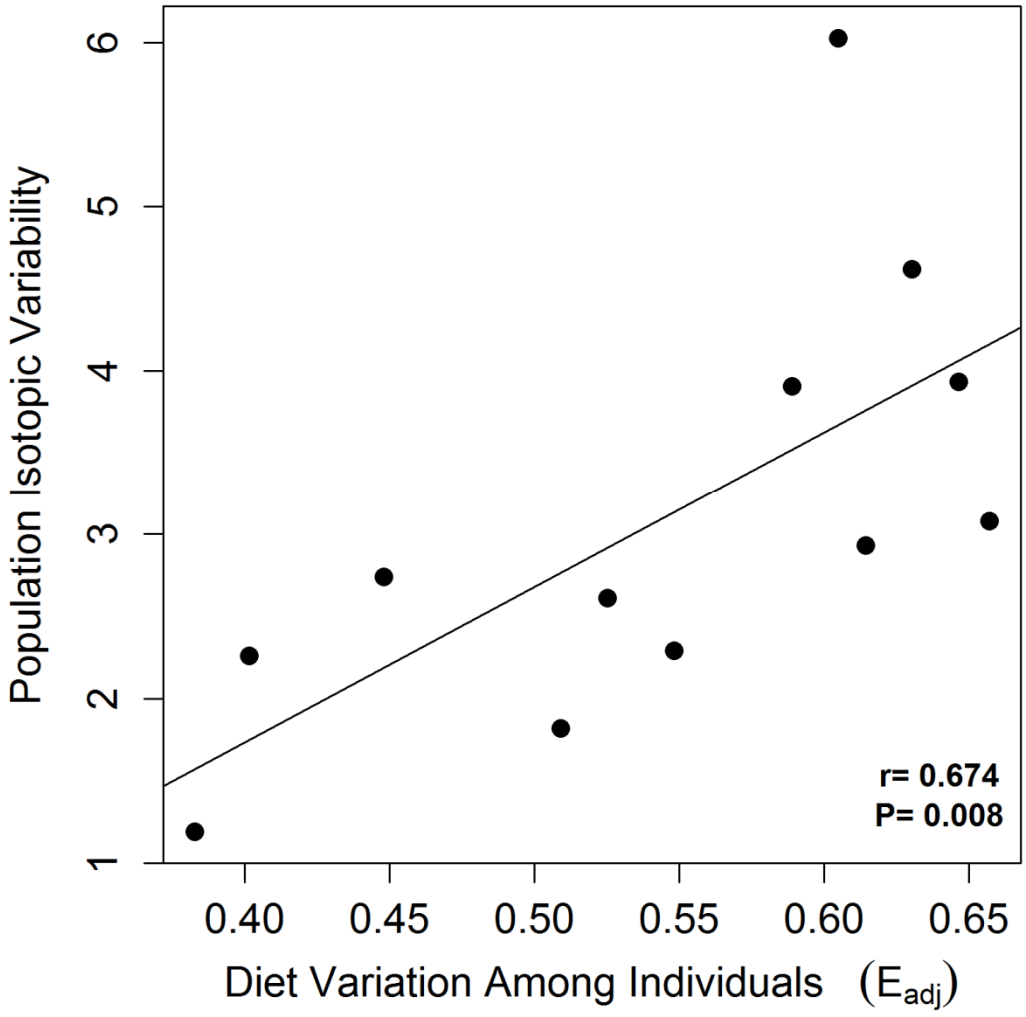

Figure 2.4: The relationship between amount of diet variation among individuals within a population(E_{adj}) and amount of isotopic variability (measured as sum of eigenvalues of PCA of $\delta^{13}C$ and $\delta^{15}N$). Each point represents a separate lake population. Pearson's correlation coefficient (r) and one-tailed P-value are provided.

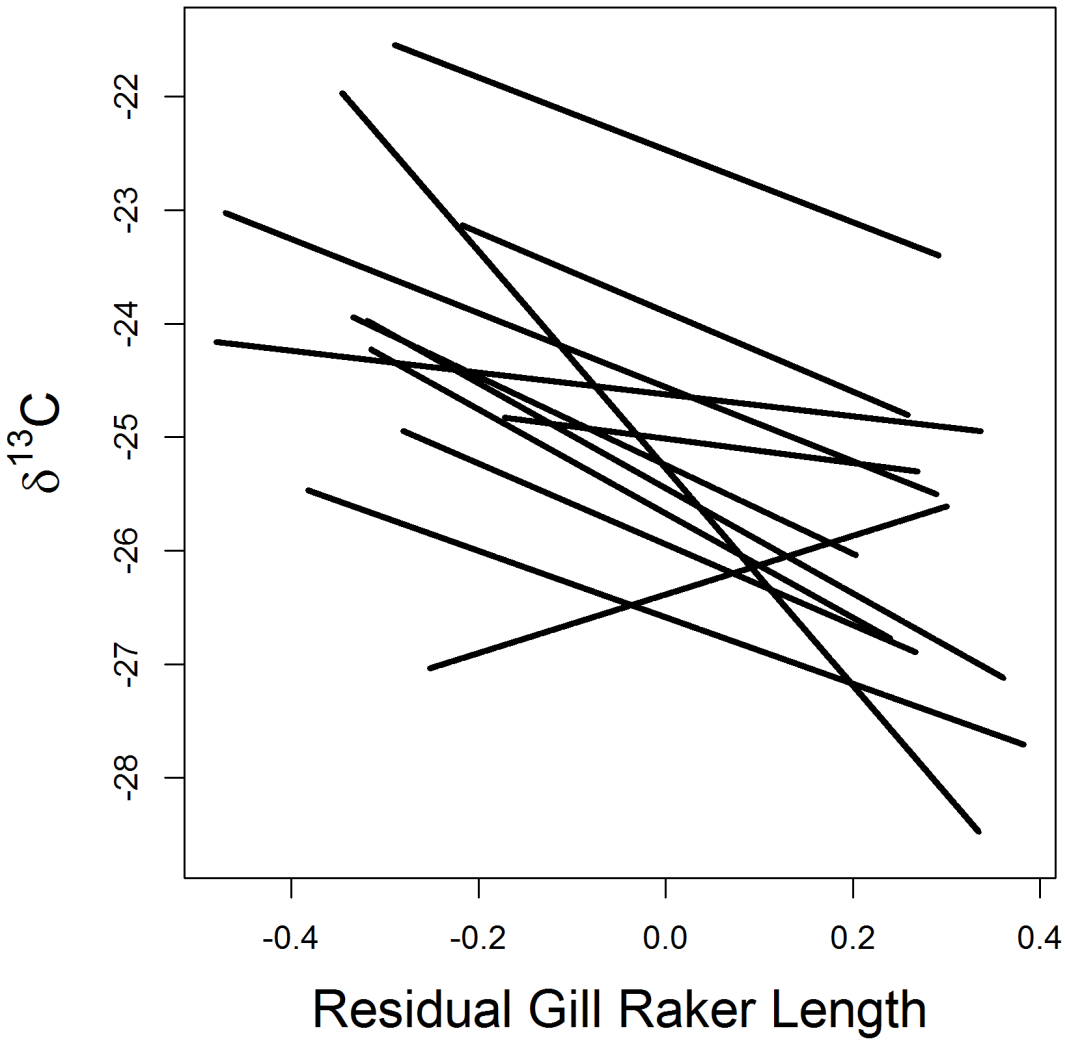

Figure 2.5: Illustration of a significant two-way morphology by population interaction for the relationship between δ13C and residual gill raker length. The global relationship can be visualized as a general negative relationship where fish with longer relative gill raker length have a more limnetic carbon signature (lower δ13C value). The lake interaction can be visualized by the different slopes of different lines with each line representing a population. Each line represents the trend for a single population within this study.

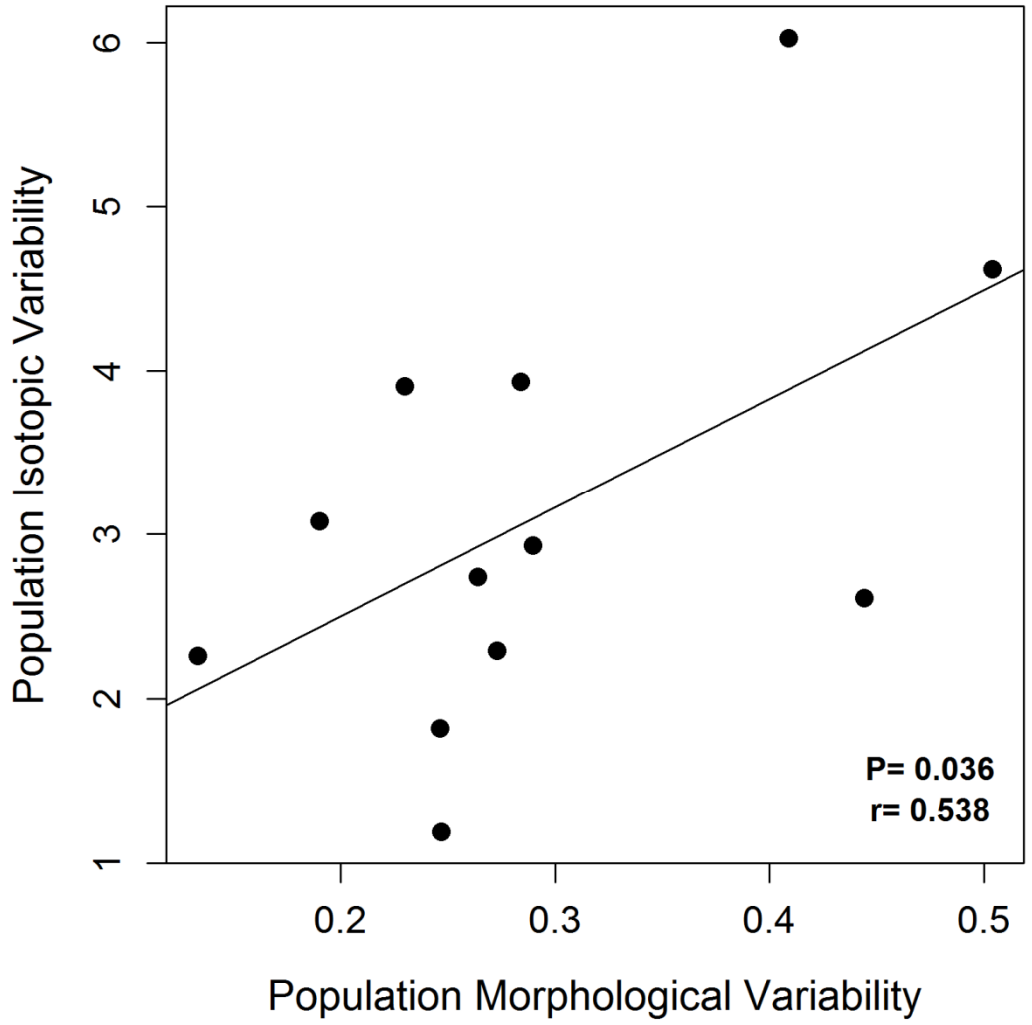

Figure 2.6: The relationship between amount of morphological variability (measured as sum of eigenvalues of PCA including scaleless morphological characters) and amount of isotopic variability (measured as sum of eigenvalues of PCA of $\delta^{13}C$ and $\delta^{15}N$). Each point represents a separate lake population. Pearson's correlation coefficient (r) and one-tailed P-value are provided.

Chapter 3

Ecologically based assortative mating in a non-diverged population of stickleback [1]

3.1 ABSTRACT

Speciation with gene flow may be driven by a combination of positive assortative mating and disruptive selection, particularly if selection and assortative mating act on the same trait, reducing recombination between ecotype and mating type. Phenotypically unimodal populations of threespine stickleback (*Gasterosteus aculeatus*) are commonly subject to disruptive selection due to competition for alternate prey. Here we present evidence that stickleback also exhibit assortative mating by diet. Among-individual diet variation leads to variation in stable isotopes, which reflect prey use. We find a significant correlation between the isotopes of males and eggs within their nests. Because egg isotopes are derived from females, this correlation reflects assortative mating between males and females by diet. In concert with disruptive selection, this assortative mating should facilitate divergence. However, the stickleback population remains phenotypically unimodal, highlighting the fact that assortative mating and disruptive selection do not guarantee evolutionary divergence and speciation.

3.1.1 Keywords:

stable isotopes, individual specialization, speciation, reproductive isolation

[1]Large portions of this chapter have been previously published as: Snowberg, L., and D. I. Bolnick. 2008. Assortative mating by diet in a phenotypically unimodal but ecologically variable population of stickleback. American Naturalist 172:733-739.

3.2 INTRODUCTION

Positive assortative mating takes place when individuals mate with individuals who are like themselves morphologically or behaviorally. Assortative mating has been recognized as an important evolutionary force, creating or maintaining linkage disequilibrium between loci, and Hardy-Weinberg disequilibrium within loci. Under certain conditions, theory suggests that assortative mating can establish enough disequilibrium to drive speciation between potentially interbreeding populations (Maynard Smith 1966; Kirkpatrick and Ravigné 2002; Gavrilets 2004). In particular, any disequilibrium arising from ecologically-driven disruptive selection may be amplified by assortative mating. Eventually this process may lead to morphologically divergent groups that, due to assortative mating, are reproductively isolated. Sympatric speciation is most likely when a single trait is both under disruptive selection and is the basis of assortative mating (Udovic 1980; Dieckmann and Dobeli 1999, Fry 2003). Such a scenario increases the probability of speciation by eliminating recombination between the trait under divergent selection and the trait used in assortative mating (Felsenstein 1981).

Some skeptics of sympatric speciation have questioned whether traits under divergent selection are commonly the basis of assortative mating. Such traits have therefore been dubbed "magic traits" to highlight their uniquely favorable effect on speciation and possible rarity (Gavrilets 2004, 2005). However, there are some clear instances of ecological divergence directly causing assortative mating. Ecological divergence may lead to allochronic isolation, as in cases where plants grow on different soil types that favor different flowering times (Savolainen et al. 2006) or for insects whose host plants fruit or bud at different times (Feder and Filchak 1999; Groman and Pellmyr 2000). Ecological divergence may also contribute to reproductive isolation when habitat preferences lead to spatial segregation of mating pairs, as occurs when divergent

host races of phytophagous insects mate on their host plants (Caillaud and Via 2000; Berlocher and Feder 2002). Adaptive morphological divergence can also serve as a basis of assortative mating. For example, size is an important ecological character in many species, and size differences also contribute to reproductive isolation in sympatric morphs of some fish species (McKinnon et al. 2004). While these examples provide some evidence for the applicability of magic trait models of assortative mating, many of these instances of assortative mating are drawn from already divergent host races or incipient species pairs with distinctly bi- or multi-modal trait distributions. In such cases, it is not clear whether the assortative mating preceded, accompanied, or followed the ecotypic divergence. For assortative mating to facilitate divergence there must be some non-random mating within single populations prior to divergence. It would therefore be valuable to look for instances of assortative mating based directly on ecological parameters themselves, in a population lacking morphological or behavioral clusters. The combination of disruptive selection and assortative mating on a single trait should quickly lead to divergence under some conditions. However, theoretical models suggest that these forces must be fairly strong to drive polymorphism or speciation, otherwise they may simply act to maintain genetic variation in a phenotypically unimodal population (Bolnick 2006; Bürger et al. 2006).

One place to look for assortative mating by a trait under disruptive selection is in lacustrine populations of the threespine stickleback (*Gasterosteus aculeatus*). Stickleback are best known for the few lakes with sympatric species pairs that exhibit strong ecological and morphological differences and assortative mating (Schluter and McPhail 1992). However, most lake stickleback occur in morphologically unimodal 'solitary' populations. In these populations, stickleback may use either benthic or limnetic prey (Schluter and McPhail 1992), and individuals differ in their propensity to use these

alternate resources (Svanbäck and Bolnick 2007; chapter two). As in the species-pair lakes, fish with larger gapes, deeper bodies, and fewer, shorter gill rakers are more efficient at using benthic prey, whereas the opposite is true for limnetic prey (Robinson 2000). These unimodal morphological traits are commonly subject to disruptive selection (Bolnick and Lau, 2008), apparently due to intraspecific competition for alternate prey (Bolnick 2004a). This disruptive selection might indirectly promote assortative mating between individuals with similar morphology and resource use (Doebeli et al. 2007).

Assortative mating by diet may take place if stickleback use some cue to evaluate prospective mates or if assortative mating is a passive consequence of another preference, such as habitat choice. Recent laboratory experiments suggest that stickleback can directly assess the diet of other individuals (Ward et al. 2004). The precise mechanism for assessment is unknown, but it appears to be based on olfactory cues (Ward et al. 2004). Regardless of the mechanism, stickleback's preference for conspecifics with similar diets might lead to assortative mating within an ecologically heterogeneous population, amplifying the effects of disruptive selection arising from competition for alternative prey (Bolnick 2004a). We therefore tested for diet-based assortative mating in a wild population of three-spine stickleback, using stable isotopes as a measure of diet.

Stable isotope ratios of individuals reflect isotope signatures of their prey over the period that the tissue was synthesized (Hobson and Clark 1992a), and are therefore commonly used to infer diet (Tieszen et al. 1983). Carbon and nitrogen isotopes provide complementary information on prey. Carbon isotope ratios differ between benthic and limnetic prey (France 1995). Nitrogen isotope ratios display a stepwise enrichment at each trophic level (Hobson and Clark 1992b). When individual stickleback vary in their propensity to consume benthic versus limnetic prey, their stable isotope ratios vary accordingly. We present evidence for assortative mating by diet, based on correlations

between the isotopes of males, and the eggs in their nests. Because egg isotopes are correlated with female isotopes, a correlation between males and the eggs in their nests implies a correlation between the isotope signatures of mated pairs.

3.3 MATERIALS AND METHODS

To test for assortative mating based on diet similarity we collected male stickleback that were guarding nests and the eggs from within those nests. We conducted this study over a five day period during June 2007 in Mohun Lake, British Columbia (50˚ 9' 49" N, 125˚ 29' 17" W). Snorkelers identified nuptial males and their nests by observing male behavior. Males return to their nests regularly and fan the nest with a characteristic head down position. We collected 41 males and the eggs from their nests using small aquarium nets. We also collected 19 gravid females from the same population using minnow traps. Fish and eggs were frozen in liquid nitrogen for later stomach content analysis, isotopic analysis, and measurement.

We measured the $\delta^{13}C$ and $\delta^{15}N$ isotope ratios of the muscle of males and females and the eggs collected from male nests and females' ovaries. $\delta^{13}C$ and $\delta^{15}N$ represents the ratio of the uncommon heavy isotope (^{13}C or ^{15}N) to the more common light isotope (^{12}C or ^{14}N), adjusted to an international standard, and are reported in parts per thousand. We used the isotopes of eggs collected from a male's nest as a proxy for the isotopes of the female which he mated with. Egg isotopes have been shown to be correlated with the isotopes of the female in fish (Gray 2001), and we confirmed this result by evaluating the correlation between females' isotopes and isotopes of eggs from their ovaries. We used one to two eggs from each nest for stable isotope analysis.

To evaluate whether isotope variance in the wild caught fish exceeds expectations

under a null hypothesis of a similar diet across individuals, we compared isotope variation in our sample to isotope variation in lab-reared fish fed a shared diet. Lab reared fish were F1 from wild-caught parents. Eggs were hand-fertilized and young were raised at 17 ˚C on brine shrimp, and then switched to freeze-dried bloodworms after reaching 1 cm standard length. We sacrificed 22 individuals from different families for isotope analysis at nine months of age.

Benthic and limnetic prey vary in both $\delta^{13}C$ and $\delta^{15}N$ ratios, with the result that benthic/limnetic diet variation in stickleback generates a correlation between these isotopes in fish tissues. We therefore performed a PCA on isotope ratios and used the first principal component axis as a measure of benthic versus limnetic feeding history. We then tested for assortative mating by evaluating the correlation between isotope PC1 scores for males and the eggs from their nests. Significance of the correlation between male and egg isotopes was evaluated parametrically with Pearson's r. To evaluate how robust the parametric result is, we also ran a non-parametric permutation test in R (R Development Core Team 2012) in which we shuffled egg isotopes without replacement 10,000 times, and determined the distribution of null values for the correlation coefficient and how often null values were more extreme than the observed one.

A correlation between the isotope ratios of males and the eggs in their nests would demonstrate a correlation between male and female isotope ratios (and hence assortative mating). In theory, if the correlation between eggs and females is P_{ef}, and the correlation between females and males is P_{fm}, then the correlation between eggs and males is $P_{ef}*P_{fm}$. This assumes that the relationships between eggs and females and between females and males are both linear (Sokal and Rohlf 1994), and that male isotopes predict egg isotopes only through their correlation with female isotopes. The correlation between males and females can then be estimated as the correlation between eggs and males divided by the

correlation between eggs and females. The correlation between male and egg isotopes was also evaluated using canonical correlation analysis, which yielded qualitatively similar results. We present the PC1 correlations here because the axis is intuitive, and because it permits us to estimate the underlying male-female correlation and to apply an ANCOVA testing for sex-dependent isotope-morphology correlations.

To test for morphological correlates of isotope variation, we thawed and blotted dry each specimen, and recorded mass (to 0.01 g), standard length, and open gape width (using digital calipers accurate to 0.01 mm). We also counted gill raker number under a dissecting microscope and measured the length of the longest gill raker using an ocular micrometer. We log-transformed mass, standard length, gape width, and gill raker length. We used these log-transformed variables along with gill raker number to perform a principal components analysis (PCA) of morphology. We used a linear model to test for a relationship between morphology and isotopes. We used sex and morphological PC1 and PC2 as independent variables (with sex* PC interactions) and isotope PC1 for fish muscle as the dependent variable.

To test for diet variation directly, we identified the stomach contents of each individual to the lowest feasible taxonomic level. We quantified the degree of among-individual niche variation in the population and the degree to which niche variation reflects dietary clusters using the program DIETA1 (Araújo et al., 2008). Interindividual niche variation (*E*) ranges from 0 (no individual niche variation) to 1.0 (no overlap in diet between any pairs of individuals; Araújo et al., 2008). The clustering index (*C*) measures the degree to which the population is organized into discrete groups of individuals sharing a common prey niche and overlapping little with the prey niches of other groups, with a value of 0 representing no clustering, $C = 1$ indicates maximal clustering, and C = -1 indicates overdispersed diet variation (Araújo et al., 2008). Both indices were tested

against a null hypothesis that individuals sampled randomly from a shared diet distribution, using a Monte Carlo resampling routine implemented in DIETA1.

3.4 RESULTS

Morphology was distributed unimodally among the fish sampled. Despite the lack of discrete morphological groups, there was a high degree of interindividual variation in diet. On average, two randomly chosen individuals' stomach contents were 70% different (E= 0.7101; P< 0.0001 for the Monte Carlo test of the null hypothesis E= 0, that individuals sample randomly from a shared prey frequency distribution). This diet variation is quantitatively very similar to that observed in previous studies of stickleback (Araújo et al, 2008). However, this diet variation was not organized into discrete clusters (C= -0.0294, P=1.0). Diet variation was reflected in isotope variances: wild-caught fish had significantly more variable isotope signatures than a sample of laboratory-bred stickleback of unknown sex raised on a shared diet (lab reared fish: N= 22, var $\delta^{13}C$= 0.1772, var $\delta^{15}N$= 0.0520; wild-caught males: N= 41, var $\delta^{13}C$= 2.301, $F_{40, 21}$= 12.98, P< 0.001, var $\delta^{15}N$= 0.186, $F_{40, 21}$= 3.58, P= 0.0013; wild-caught females: N= 19, var $\delta^{13}C$= 3.412, $F_{18, 21}$= 19.25, P< 0.001, var $\delta^{15}N$= 0.201, $F_{18, 21}$= 3.87, P= 0.0019).

The eggs from males' nests reflect the range of isotope signatures seen in eggs collected from females. There was some evidence of $\delta^{13}C$ and $\delta^{15}N$ depletion when we compared females' isotopes to eggs from their own ovaries. Compared to the female they were harvested from, eggs showed a mean $\delta^{13}C$ depletion of 1.79 ‰ and a mean $\delta^{15}N$ depletion of 1.78 ‰ (paired t-tests, $\delta^{13}C$: t_{18}= 5.595, P< 0.001, $\delta^{15}N$: t_{18}= 21.627, P< 0.001). The isotopic variances did not differ between females and their eggs ($\delta^{13}C$: $F_{18, 18}$= 1.761, P= 0.24, $\delta^{15}N$: $F_{18, 18}$= 0.657, P= 0.38).

There was a correlation between $\delta^{13}C$ and $\delta^{15}N$, arising from associations between these signatures in benthic versus limnetic prey. This axis of isotope variation matches that observed between benthic and limnetic species pairs (Matthews et al. 2010), and between fish in parapatric benthic and limnetic habitats (Bolnick et al, 2008). We therefore used the first principal component of isotope variation (λ_1= 1.19, percent of total variance explained= 59.43 %) to represent benthic versus limnetic feeding history. We did not directly test for correlations between stomach contents (e.g., percent benthic prey) and stable isotope signatures. Our stomach content data was in the form of counts, and due to time constraints we did not collect the prey mass data required to convert these into a measure that would be comparable with isotope signatures. Previous studies have shown that gut contents are correlated with muscle stable isotopes in solitary lacustrine stickleback populations (Bolnick et al, 2008).

Using the first principal component of isotope variation we found that there is a significant correlation between isotopes of females and their eggs (Figure 3.1A, R= 0.687, P = 0.001). This confirms that eggs may be used as a proxy for females, to test for assortative mating (male-female correlations). Consequently, the significant correlation between the isotopes of males and the eggs in their nests (Figure 3.1B, R= 0.348, P= 0.012 with the parametric test, and P = 0.014 with the permutation test), can be used to infer that there is a correlation between male and female isotope PC1. This implies that more benthic-feeding males tend to mate with benthic-feeding females (and limnetics with limnetics) more often than expected by chance. Using the observed correlation between male and egg isotopes and between female and egg isotopes we estimate the male-female correlation as 0.507.

Log-transformed mass, standard length, and gape width loaded on morphological PC1. Raker number and log-transformed raker length loaded on morphological PC2

(Table 3.1). Both morphological PC1 and PC2 were correlated with isotope PC1 (Table 3.2). Larger fish tended to exhibit a more benthic isotope signature. Fish with fewer and shorter gill rakers also tended to exhibit a more benthic isotope signature. Morphology*sex interaction terms represent a difference in slope but not a difference in trend in the relationship between morphology and isotopes in males and females (Figure 3.2). The effect of morphological PC1 and morphological PC2 on isotope PC1 was stronger for females than males.

3.5 DISCUSSION

Stickleback exhibit strong within-population diet variation, or individual specialization (Svanbäck and Bolnick 2007). Individuals vary in their propensity to consume benthic versus limnetic prey, even when held in small (10 m^2) enclosures that ensure all individuals have access to the same set of prey (Araújo et al., 2008). Because benthic and limnetic prey differ in their stable isotope ratios, diet variation is reflected in isotopic variation among individuals. This isotope variation is consistently correlated with morphology within populations, confirming that among-individual diet differences persist for significant lengths of time. Isotope variance in wild-caught fish was an order of magnitude higher than what we observed when all individuals were reared on the same resource, reflecting prey variation in the wild-caught fish.

Experimental studies of shoaling behavior, which utilized diet manipulations, have shown that individual stickleback preferred to associate with conspecifics that fed on similar prey, suggesting that diet *per se* is involved in association behavior (Ward et al. 2004). We posited that shoaling preference might carry over to cause assortative mating by diet in ecologically heterogeneous populations of stickleback. Such assortative

mating could be detected as a correlation between the isotope signatures of males and females. Using egg isotopes as a proxy for females, we have demonstrated that such a correlation exists, and we may thus conclude that stickleback in Mohun Lake do exhibit some assortative mating. This population is phenotypically unimodal and diet variation was not in discrete clusters, so this assortative mating occurs within a single population rather than representing reproductive isolation between divergent 'morphs'.

Mate choice and assortative mating have been studied extensively in stickleback, especially in populations characterized by separate benthic and limnetic groups. Assortative mating between these groups has been demonstrated based on size (Nagel and Schluter 1998; Vines and Schluter 2006) and nuptial color (McKinnon 1995; Boughman 2001). However, our results present the first evidence of assortative mating within a phenotypically unimodal population of stickleback. Assortative mating by diet in unimodal populations represents the first example of a potential 'magic trait' in stickleback, although more work is necessary to determine whether disruptive selection and assortative mating truly act on the same trait or whether they act on traits that are correlated.

While we found a correlation between male and female isotopes, there are a number of mechanisms that might drive the underlying assortative mating. It is possible that individuals select mates directly based on olfactory cues associated with recently consumed prey, as suggested by studies of shoaling in laboratory aquaria (Ward et al. 2004). Alternatively, stickleback could be selecting mates based on morphological traits that are correlated with diet. We found that isotope PC1 was correlated with size (standard length, mass, and gape width) and gill raker traits (length and number). Assortative mating could be based on size, as is commonly found in fish with different morphs (Foote and Larkin 1988; Nagel and Schluter 1998; McKinnon et al. 2004). Gill

raker traits, being internal, are unlikely to be direct targets of mate choice.

Finally, it is possible that the isotope correlations arose from spatial heterogeneity. This spatial effect may be of two types. First, baseline isotope signatures may vary spatially. If stickleback exhibit strong philopatry, then there may be spatial gradients in isotope signatures in both males and females, leading to the appearance of assortative mating. However, all our nests were collected along approximately 250 m of homogeneous shoreline. Mark-recapture studies show that stickleback can move that distance within a few days (Bolnick et al. 2009), so isotopes are unlikely to vary dramatically over such a small distance. Second, spatial effects may arise if individuals that feed on different prey tend to select different microhabitats for mating. In species pairs lakes, benthic and limnetic stickleback differ in their nest location and characteristics (McPhail 1994). Benthic-like and limnetic-like populations from solitary lakes also differ in their nest location (Vines and Schluter 2006), but differences within solitary populations have not been shown. Hence, nest-site selection might be an effective basis for assortative mating that could generate correlations between isotope signatures.

Regardless of whether mate choice is based on diet itself, or morphology, or microhabitat that is correlated with diet, the ultimate outcome is assortative mating with respect to diet. Assortative mating based on a trait directly under disruptive selection is the most favorable situation for speciation in the presence of gene flow (Maynard Smith 1966; Felsenstein 1981; Dieckmann and Doebeli 1999; Kirkpatrick and Ravigné 2002; Fry 2003). Disruptive selection is common in solitary lacustrine populations of stickleback (Bolnick and Lau 2008), acting on gill raker traits that are correlated with isotopes in the present study. This should be a favorable situation for disruptive selection and assortative mating to lead to speciation. However, both Mohun Lake stickleback and all the populations in surrounding lakes remain phenotypically unimodal and are in

Hardy-Weinberg equilibrium (Caldera and Bolnick 2008). This result leaves us with an interesting puzzle: models suggest that sympatric speciation is easiest when disruptive selection and assortative mating act in concert, yet we find no indication of divergence in stickleback despite the joint action of these processes. We propose that the strength and/or temporal consistency of assortative mating and disruptive selection in these populations may be insufficient for speciation to proceed (Bolnick 2004b, 2011).

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Axis	λ_i	% Var	SL	Mass	GW	GRL	GRN
PC1	2.669	53.39	0.965	0.954	0.885	0.196	0.082
PC2	1.385	27.70	-0.160	-0.190	0.127	0.790	0.826

Table 3.1: Principal component analysis of fish morphology. Principal component analysis results showing the eigenvalues (λ_i), the percentage of total variance explained, and the component loadings for morphology (SL= log-transformed standard length, Mass= log-transformed body mass, GW= log-transformed gape width, GRL= log-transformed length of longest gill raker, GRN= number of gill rakers).

Source	SS	df	MS	F	*P*
Morphology PC1	2.314	1	2.314	16.501	<0.001
Morphology PC2	2.224	1	2.224	15.861	<0.001
Sex	1.597	1	1.597	11.389	0.001
Morphology PC1*Sex	1.108	1	1.108	7.9	0.007
Morphology PC2*Sex	0.588	1	0.588	4.19	0.046
Error	7.573	54	0.14		

Table 3.2: Linear Model Results. Summary of linear model results showing the source of variation, sum-of-squares, degrees of freedom, mean square, F-ratio, and significance value with isotope PC1 as the dependent variable. The PC1 x PC2 interaction and three-way interactions are not significant ($P > 0.9$) and for brevity are not included.

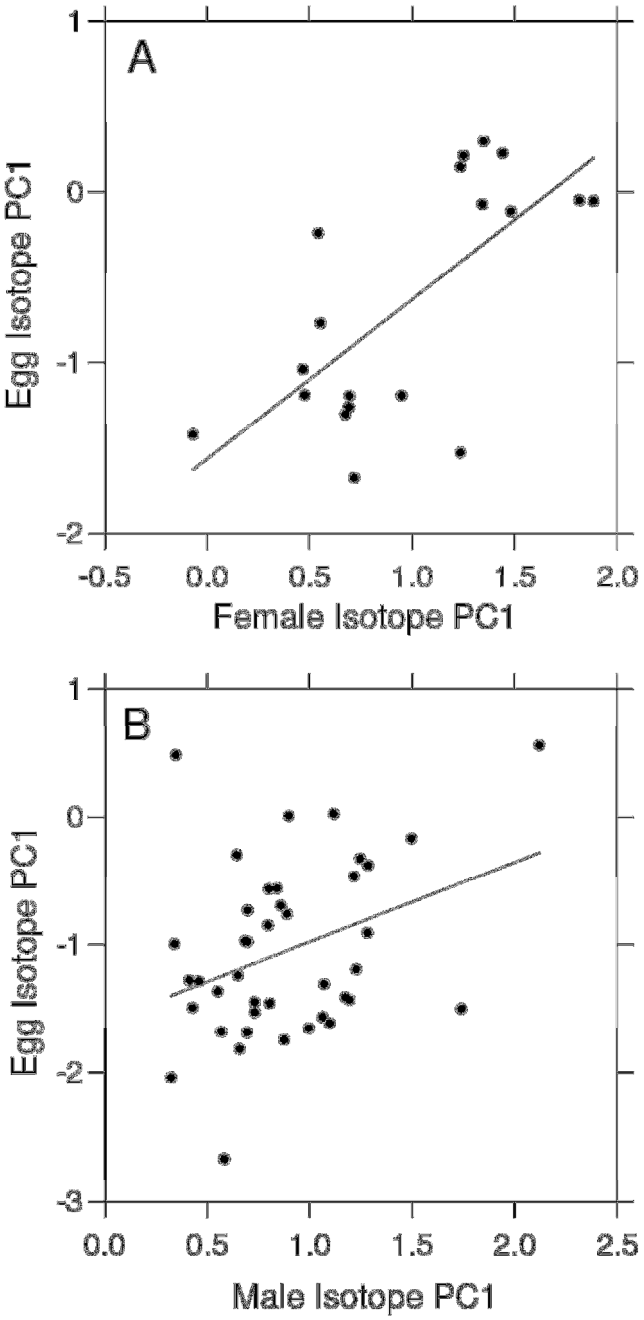

Figure 3.1: The correlation between the first principal component of isotope variation between females and their eggs (A) and males and the eggs collected from their nests (B).

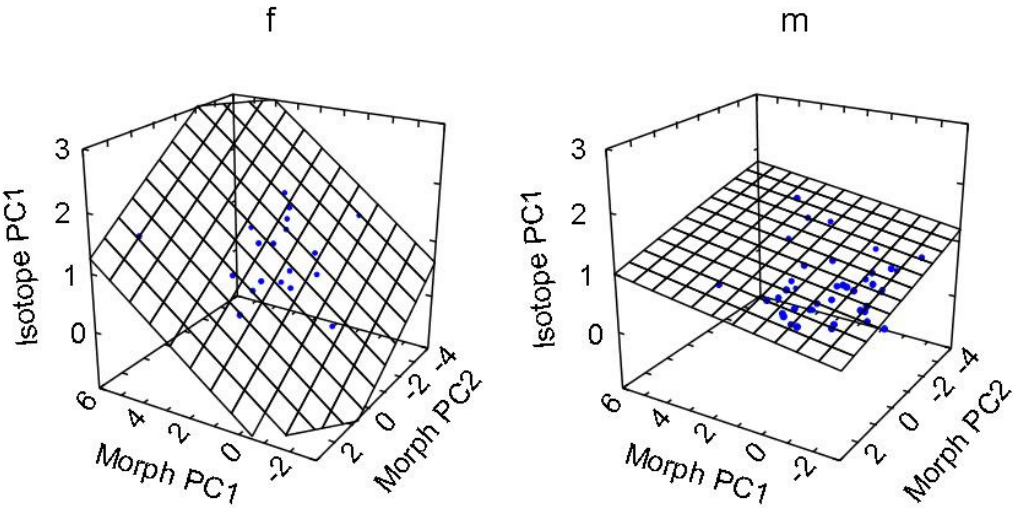

Figure 3.2: The relationship of Morph PC1 (size) and Morph PC2 (gill raker traits) with Isotope PC1 for female and male stickleback.

Chapter 4

Individual diet, not habitat isolation, causes ecologically based assortative mating within a population of threespine stickleback [2]

4.1 ABSTRACT

Assortative mating is measured as a phenotypic or genotypic correlation between mates. Although biologists typically view assortative mating in terms of mate preference for similar partners, correlations between mates can also arise from phenotypic spatial structure arising from spatial isolation or habitat preferences. Here, we test whether diet-assortative mating within an ecologically variable population of threespine stickleback results from small-scale geographic isolation or microhabitat preference. We find evidence for assortative mating in the form of a positive correlation between mated pairs' diets (measured using stable isotopes). Stable isotopes reveal diet differences between different nesting areas and among individuals using different nest habitat within a nesting area. This spatial segregation of diet types should generate some assortative mating, but is insufficient to explain the observed assortment strength. Significant male-female isotope correlations remain after controlling for spatial variables. We therefore conclude that sticklebacks' diet-assortative mating arises from additional behavioral preference. More generally, our results illustrate the point that spatial segregation can only drive appreciable levels of phenotypic assortative mating when environment-phenotype

[2]Large portions of this chapter have been previously published as: Snowberg, L., and D. I. Bolnick. 2012. Partitioning the effects of spatial isolation, nest habitat, and individual diet in causing assortative mating within a population of threespine stickleback. Evolution 66:3581-3594.

correlations are parallel and strong in both sexes. Consequently, intraspecific assortative mating may typically entail mating preferences rather than just spatial co-segregation of phenotypes.

4.1.1 Keywords

Gasterosteus aculeatus, habitat choice, diet variation, stable isotopes, spatial cosegregation

4.2 INTRODUCTION

Assortative mating occurs when there is a phenotypic or genotypic correlation between mates (Wright 1921). Such correlations may drive deviations from Hardy-Weinberg equilibrium, inflate the genetic variance of a population (Lynch and Walsh 1998), and promote reproductive isolation between populations (Felsenstein 1981; Coyne and Orr 2004). The resulting deviations from Hardy-Weinberg equilibrium can also create statistical biases in quantitative genetics and association mapping studies (Gimelfarb 1986; Falconer and Mackay1994; Redden and Allison 2006). Assortative mating is therefore of interest when studying the genetic structure of populations or speciation.

Despite its importance for population genetics, the mechanistic basis of assortative mating is often unknown. Assortative mating may arise from multiple mechanisms including (1) mating preference for phenotypically similar individuals (Andersson 1994), (2) directional sexual selection on both sexes (Crespi 1989), or (3) spatial or temporal structure of phenotypes during the breeding season (Rice 1987). The first two of these mechanisms both require the expression of mating preferences for

specific phenotypic traits. In contrast, spatially-generated assortative mating does not require mating preferences, instead assortment arises incidentally because phenotypically similar individuals are more likely to encounter each other. Consequently, if biologists are to understand the role of mate preferences in driving assortative mating, they must also evaluate the alternative hypothesis that assortative mating is an artifact of spatial heterogeneity.

Two major types of spatial heterogeneity can generate trait correlations between mated pairs. First, dispersal barriers or isolation by distance may simultaneously promote phenotypic spatial divergence, and constrain individuals to mate locally. Phenotypic divergence between geographically distinct sub-populations may be due to adaptive genetic differences or phenotypic plasticity. As long as individuals are more likely to mate with spatially proximate individuals, this spatial phenotypic structure will lead to assortative mating even in the absence of behavioral preferences for mates with specific traits.

Second, divergent phenotypes may be spatially well-mixed, but exhibit different behavioral microhabitat preferences, such that individuals are more likely to encounter similar individuals when mating, but be well mixed at all other times in their life history. Assortative mating is again a result of reduced encounter rate between individuals of different phenotypes, but at a fine spatial scale that might not be immediately obvious. Behavioral choices may indeed play an important role in assortative mating by habitat choice, but individuals choose their breeding location rather than their partner's phenotype.

The role of discrete habitats in assortative mating has been recognized for some time. For example, ecological divergence can contribute to reproductive isolation when habitat preferences lead to spatial segregation of mating pairs, as occurs when divergent

host races of phytophagous insects mate on their host plants (Caillaud and Via 2000; Berlocher and Feder 2002). More generally, assortative mating can occur whenever male and female phenotypes influence the choice of breeding site or time. If both male and female morphology are correlated with breeding location, a correlation between the morphologies of mated pairs may occur even in the absence of mate preferences. Such habitat-induced correlations are obvious when they involve mating in discrete habitat types, but may be overlooked when they arise through fine-scale microhabitat partitioning. Despite hundreds of empirical studies of within-population assortative mating in animals (Jiang, Bolnick, and Kirkpatrick, manuscript), very few, if any, distinguish between spatial structure and preference in generating assortment.

Here, we describe a test of whether spatial cosegregation underlies assortative mating in an ecologically variable but phenotypically unimodal (single-species) population of threespine stickleback (*Gasterosteus aculeatus*). Snowberg and Bolnick (Chapter 3) documented assortative mating with respect to diet (measured by stable isotopes) in one population of phenotypically variable stickleback. We confirm this previous finding in another stickleback population, and test whether this assortment arises from spatial structure between phenotypes and breeding location or microhabitat. Based on our results, we offer some general insights as to how readily spatial structure can generate intraspecific assortative mating.

4.2.1 Study system

Threespine stickleback are a model system in ecology, evolution, and behavior. Many studies have focused on reproductive isolation between sympatric benthic/limnetic species pairs (Boughman 2001; Rafferty and Boughman 2006; Kozak and Boughman

2009; Kozak et al. 2011), divergent lake/stream (Raeymaekers et al. 2010; Eizaguirre et al. 2010), marine/freshwater (Bell 1982; McKinnon et al. 2004), and marine/marine ecotypes (Kitano et al. 2009; Kume et al. 2010). Recently, Snowberg and Bolnick (Chapter 3) also found evidence for assortative mating within populations, in which mated male/female pairs were more ecologically similar than expected by chance. Within any given lake population of stickleback, individuals differ in their propensity to eat benthic macroinvertebrates or pelagic zooplankton, as revealed by stomach content analyses, isotopic measures of diet, and direct observation of foraging microhabitat use (Araujo et al. 2008; Bolnick and Paull 2009; Matthews et al. 2010; chapter two). Individual diet is also consistently associated with trophic morphology (chapter two). Snowberg and Bolnick (Chapter 3) found a correlation between male and female isotopes from mated pairs, indicating assortative mating with respect to diet (which is reflected in isotope ratios, see below). However, it is unknown whether this assortative mating is a result of mate preferences for diet-derived cues, morphological traits correlated with diet, or because diet types are spatially segregated across a lake or among adjoining microhabitats. Specifically, the previously observed assortative mating could be a result of spatial isolation: across-lake gradients in stickleback diets, such that ecologically divergent individuals are less likely to encounter each other during the breeding season. Or, assortative mating could reflect micro-habitat preferences: individuals with divergent diets may be spatially well mixed and frequently encounter each other, but select subtly different nest sites when mating. This microhabitat partitioning may reduce interbreeding among diet strategies even when they are in close spatial proximity.

There are a number of prior studies suggesting that ecological difference may be associated with nesting habitat. In a few lakes, stickleback exist as sympatric species pairs (benthic and limnetic species), which exhibit strong ecological, morphological, and

genetic differences that are sustained by assortative mating (Schluter and McPhail 1992). Benthic and limnetic stickleback differ in their nest location and characteristics, with limnetic males nesting in open, shallower areas and benthic males nesting in dense vegetation at deeper depths within the littoral zone (McPhail 1994). Females also differ in their habitat use, making encounters with males of their own species more likely for benthic and limnetic stickleback (Vamosi and Schluter 1999). Benthic-like and limnetic-like populations from allopatric solitary lakes also differ in their nest location in a manner similar to the species pairs (Vines and Schluter 2006). Given this background information, we posit that ecologically divergent individuals within a solitary population may tend to select different microhabitats for mating, leading to assortative mating. Consistent with this possibility of assortment via spatial structure, we have found some morphological, isotopic, and dietary differences among sites within a given lake, though most (~90%) trait variance occurs within rather than among collection sites within a lake (Snowberg, Bolnick, and Paull, unpub data). Even within a site, individuals caught in adjoining traps (meters apart) tend to exhibit significantly different isotopes, suggesting micro-scale habitat choice or assortative shoaling that could generate spatially-driven assortative mating. Finally, experimental transplants show that individuals can actively choose habitats based on their phenotype, facilitating adaptive divergence (Bolnick et al. 2009).

The primary goals of this study are to determine if (1) there is assortative mating within a single population of stickleback, as indicated by a correlation between the diets of mated individuals, (2) there is spatial isolation between individuals with different isotopes (diets), (3) there is microhabitat segregation by diet?, and (4) geographic and microhabitat segregation is sufficient to explain the observed assortative mating. If not, we must invoke additional sources of non-random mating (most likely mate preferences)

to explain assortative mating within populations. In addition, we test for divergence in male trophic morphology across microhabitats, which would suggest that males with different phenotypes (including diet) select different nest microhabitats (matching habitat choice; Edelaar et al. 2008), instead of nest location dictating individuals' diets. We then generalize our results beyond stickleback by identifying the conditions required for spatial segregation of phenotypes to yield empirically reasonable levels of assortative mating within populations.

4.3 METHODS

4.3.1 Using stable isotopes to study assortative mating

Stable isotopes are commonly used to study diet variation (Tieszen et al. 1983; Newsome et al. 2007). We take advantage of three facts in order to study diet-assortative mating in stickleback: (i) stable isotopes reveal individuals' past diets (see below), (ii) females' isotopes are passed down to their eggs, and (iii) females deposit their eggs in males' nests, which the male then guards until after hatching. Thus, if males and females mate assortatively with respect to diet, we should see a corresponding correlation between male and egg isotopes. Because males guard nests, we are able to record the mating location and habitat and study correlations between diet and habitat without directly observing mating, which is not practical in stickleback.

Carbon and nitrogen isotope ratios provide complementary information on fishes' diets. Limnetic and benthic primary producers (phytoplankton and periphyton respectively) fix C^{12} and C^{13} isotopes in different ratios (France 1995; Post 2002). These ratios are preserved in consumers' tissues with only slight fractionation. As a result, the ratio of these isotopes ($\delta^{13}C$) provides a measure of how much benthic or limnetic carbon

an individual uses (Matthews et al. 2010). Nitrogen provides a complementary measure of trophic position, because the ratio of N^{14} to N^{15} ($\delta^{15}N$) displays a stepwise enrichment at each higher trophic level (Hobson and Clark 1992a). Stable isotopes turn over slowly in tissues, integrating diet over the course of weeks to months (Hobson and Clark 1992b, Hobson 1993). Consequently, stable isotope differences among individuals may be used to infer sustained among-individual diet differences.

Female's isotopes ratios are correlated with the isotopes of their eggs (Chapter 3; Gray 2001). We confirmed this relationship for the studied population by capturing 33 gravid females, and analyzing the stable isotopes of their liver tissues and mature eggs (see below for isotope methods). The positive correlation between female and egg isotopes ($\delta^{13}C$ $r = 0.976$; $\delta^{15}N$ $r = 0.799$) allows us to use eggs' isotopes as a proxy for female diet.

4.3.2 Sample collection

This study took place in Burnt-Out Lake, British Columbia (Figure 4.1). Burnt-Out Lake is a small (approximately 8 ha) lake in the Browns Bay watershed on Vancouver Island. No other lakes in the watershed contain stickleback, minimizing the potential for immigration to inflate our estimates of assortment. Our previous study of diet-assortment in stickleback (Chapter 3) took place in a lake from a separate watershed, 5 kilometers away from Burnt-Out Lake. While small lakes are less subject to disruptive selection on diet (Bolnick and Lau 2008) and therefore may show weaker assortment, the choice of a smaller lake allowed us to survey a large proportion of the lake for nesting activity.

Over a one week period in late June 2008 we collected males and their eggs from

102 geo-referenced nests (see below for information about spatial distribution). Nest-guarding males and the eggs from their nest were collected by snorkelers with dip nets. Snorkelers searched along shorelines in all suitable nesting habitat in the lake and collected all observed nests containing eggs. Nest guarding males in this lake have bright red nuptial coloration which helped in locating breeding males. Males also display stereotyped nest care activities (e.g., fanning) that facilitated finding nests containing eggs obvious. Nests containing larval stickleback were not collected. While our collection of nests may not have been complete, it was not biased toward collecting in a particular habitat or depth range. Nests were collected between 0.2-2.1 m in depth. Visibility in the lake was high and no nests were observed deeper than our collection range. Gravid females were collected opportunistically using minnow traps and dip nets from the nesting areas.

We used a Trimble GeoXT GPS accurate to approximately 1 m horizontal distance to record the nest location in UTM. Before collecting a nest, we measured nest depth to within 10 cm, and photographed each nest to categorize vegetation cover (among dense vegetation= 1, in open area= 0) or large logs (directly under a submerged log= 1, not sheltered= 0). These microhabitat variables were chosen based on previous studies of association between stickleback nesting habitat and diet/morphology (McPhail 1994; Vines and Schluter 2006) and were supported as being biologically relevant to nesting stickleback by observations in the field: males nesting among dense vegetation or sheltered by a large log were less likely to swim away from their nest when approached by a snorkeler (L.K. Snowberg, personal obs.) Nest substrate was uniform throughout the study area and was therefore not included in analysis. We cannot rule out the possibility that other nest characteristics differed between males. We did not include nest structure as a covariate it represents an extended phenotype of the male, rather than a habitat

feature.

We collected liver samples for stable isotope analysis from all nesting males. For each male we randomly selected 1-2 eggs from a single clutch per nest (eggs from a clutch tend to be adhered into a clump and are at the same developmental stage) for isotope analysis. We also collected liver samples and eggs from gravid females. Samples were oven dried at 50 C for 72 hours. Approximately 125 µg of each sample was packed in tin capsules and shipped to the UC Davis Stable Isotope Facility for analysis. The facility analyzes $\delta^{13}C$ and $\delta^{15}N$ isotopes using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Individuals can differ in isotope ratios for either of two reasons: (1) if their diets are the same but their prey have different isotope ratios; or (2) if they have different diets. Consequently, across-lake gradients in environmental isotope ratios can lead to across-lake gradients in stickleback isotopes, even if stickleback diets are constant. If individuals mate locally, these across-lake isotope gradients could create a misleading male-female correlation in isotopes, which is not matched by male-female correlations in diet. Using basal primary consumers of benthic and limnetic producers (snails and mussels respectively), we can account for the isotopic variation among alternate resources to calculate an index of % benthic carbon and relative trophic position for each individual fish (Post 2002). We collected snails (N= 21) and mussels (N= 27) with GPS location data from throughout the nesting areas. We used the equations in Post (2002) to convert carbon stable isotope ratios into % benthic carbon. This procedure simply calculates where the fish carbon isotopes are relative to end-points defined by the carbon isotopes of snails (primary consumer assumed to represent 100% benthic carbon) and mussels (primary consumer assumed to represent 0% benthic carbon). The % benthic carbon

value for an individual is used along with the nitrogen stable isotope ratio to calculate relative trophic position (Post 2002). Relative trophic position is calculated as the extent to which $\delta^{15}N$ is enriched above the value expected given baseline benthic and limnetic $\delta^{15}N$ and the proportional contribution of those sources to the fish's diet (the estimated % benthic carbon).

A limitation of the Post (2002) method is that stickleback sometimes exhibit $\delta^{13}C$ values outside the range of mussel and snail $\delta^{13}C$ (Snowberg and Bolnick, unpub data). As a result, % benthic carbon may be negative or exceed 100%, leading to nonsensical estimates of relative trophic position. Here, for the few individuals whose carbon transgressed the baseline endpoints (16/102 males), we calculated relative trophic position by setting % benthic carbon to the closest endpoint (0% or 100%). In practice, this has a negligible effect on the calculated trophic position because the nitrogen stable isotopes of mussels and snails in this population are nearly identical. Thus the vast majority of variation in trophic position is a direct reflection on the nitrogen stable isotopes of each fish (r = 0.966 between $\delta^{15}N$ and trophic position) rather than any correlated effect arising from their carbon isotopes.

The strength of assortative mating by diet is calculated by first obtaining the correlation between nesting male and egg isotopes (r_{m*e}), and the correlation between gravid female and egg isotopes (r_{f*e}). The strength of assortative mating ($\hat{r}_{m*f}$) is the estimated correlation between male and female isotopes, calculated from the preceding two correlations as $\hat{r}_{m*f} = r_{m*e}/r_{f*e}$ (Chapter 3). The standard error of $\hat{r}_{m*f}$ is calculated using the relationship

$$SE(\hat{r}_{m*f}) = \hat{r}_{m*f} * \sqrt{SE(r_{m*e})^2 + SE(r_{f*e})^2}.$$

We analyzed correlations between male and egg isotopes (r_{m*e}) separately for trophic position and % benthic carbon. Unlike in our previous study of assortative mating by diet

(Chapter 3), carbon and nitrogen isotopes were not correlated in this population. Consequently, rather than testing for a male/egg correlation using an isotopic principal component axis (as we did previously), we separately tested each isotope measure for correlations between male liver tissue isotopes and the isotopes of eggs from the males nest. These correlations were calculated for both the whole lake and each shore individually. Because we lacked precise location data for gravid females (to assign local mussel or snail baselines), we calculated female-egg correlations using raw isotope values rather than % benthic carbon or trophic position. The conversion to % benthic carbon and trophic position are linear transformations of raw isotope ratios, so the lack of baseline data should not affect the female-egg correlation. All statistical analyses were carried out in R (R Development Core Team 2012).

4.3.3 Understanding the role of spatial cosegregation in assortative mating

To determine whether spatial structure explains diet-assortative mating, we tested whether shared male-habitat and female-habitat correlations explain the observed male-female isotope correlation. Finally, we tested whether assortment occurs that cannot be explained by the measured habitat variables, by obtaining residuals of male and egg isotopes with respect to spatial and habitat variables, and testing for a correlation between these residuals.

Nests were found along two opposite shorelines of the lake (Figure 4.1). We collected 81 nests from the northwest (NW) shore and 21 nests from the southeast (SE) shore. The difference in number of nests collected reflects a smaller nesting colony on the SE shore. The area along the shoreline between nest clusters consisted of marshy vegetation and no nests were found in this area. We therefore treat the shores as discrete

clusters of nests. It is approximately 100 m across the lake between these clusters and stickleback were observed swimming in the open water between nest shores. Stickleback must come to shallow water to nest or lay eggs (Wooten 1976).

There was no indication of spatial trends in isotopes along a given shoreline for either stickleback or mussels and snails (used as isotopic baseline). We therefore only used shore as a categorical variable in further analysis of spatial effects. Nitrogen isotopes differed significantly between shores for mussels (NW shore= 2.61 ± 0.13, SE shore= 2.97 ± 0.08, (mean ± SE), $P = 0.019$) but not snails (NW shore= 3.23 ± 0.14, SE shore= 3.00 ± 0.19, $P = 0.38$). Similarily, carbon isotopes differed significantly between shores for mussels (NW shore= -31.27 ± 0.11, SE shore= -31.54 ± 0.07, $P = 0.048$) but not for snails (NW shore= -26.22 ± 0.53, SE shore= -27.88 ± 0.77, $P = 0.10$). Consequently, we used mean mussel isotopes for each shore seperately and a lakewide mean for snails to calculate trophic position and % benthic carbon according to the methods of Post (2002) as described above. Analyses were also done with raw nitrogen and carbon isotope data and produced equivalent results throughout all analyses. In addition, using mean isotopes for each shore seperately for both snails and mussles did not change our conclusions.

We calculated the strength of male-female correlation (assortative mating) expected to arise from the observed habitat co-segregation for each measured habitat variable. We measured the correlations between each habitat variable and male and egg isotopes (r_{h*m} and r_{h*e}). We then calculated the expected correlation between male and egg isotopes, based on the male-habitat and egg-habitat correlation ($\hat{r}_{m*e} = r_{h*m} * r_{h*e}$). This formula is a simple application of standard partial correlations, in which an indirect phenotypic correlation between males and females arises from phenotype-habitat correlations within each sex. Note that the egg-habitat correlation could be influenced

both by female habitat preference and by female preference for spatially structured males. Therefore, the spatially predicted male-egg isotope may be an overestimate of the actual role of spatial segregation in assortative mating, because our calculation may be influenced by female preferences for spatially structured male traits. We compared the predicted assortment-by-space, to the observed level of assortative mating

Finally, we tested whether assortative mating remains after statistically removing the effect of both space and microhabitat. We generated separate linear models of the relationships between male and egg isotopes and measured habitat variables (shore, depth, vegetation and wood cover.) We then tested for a correlation between the residuals of these models for males and the eggs collected from their nest. A significant correlation between the residuals indicates that there is assortative mating on isotopes, above and beyond any correlations arising from habitat cosegregation.

4.3.4 Relationship between nest habitat and male diet and morphology

The spatial structure in male isotopes, evaluated above, might also be reflected in male trophic morphology. We measured morphological features typically associated with diet in stickleback (as described in Chapter 3) for all males. In both species-pair lakes and within solitary lakes, fish with larger gapes, deeper bodies, and fewer, shorter gill rakers are more efficient at using benthic prey, whereas the opposite is true for limnetic prey (Schluter 1995; Robinson 2000). For each nesting male collected we measured standard length and gape width using digital calipers (accurate to 0.01 mm). We counted the number of gill rakers on the right side of the gill arch and measured the longest gill raker using an ocular micrometer. Gape width and gill raker length are highly correlated with standard length so the residuals of these measures on standard length were used in

analyses.

We used general linear models to test whether nest habitat (depth and vegetative and wood cover) depend on male diet and morphology. Nest depth, vegetative cover, and wood cover were used as dependent variables, to reflect our hypothesis that male morphology influenced their choice of nest habitat. Our fish were sampled within weeks of the onset of the breeding season, and it is unlikely that males' nesting habitat could appreciably influence their morphology, via phenotypic plasticity, over such a short time scale. Similarly, nest habitat is unlikely to have had enough time to influence male isotopes. We evaluated three separate models with nest habitat characters as functions of male % benthic carbon, trophic position, standard length, gill raker number, and residual gill raker length and gape width. We used the function stepAIC in the MASS package of R (Venables and Ripley 2002) to chose the best fit model. For depth we fit a linear model starting with all morphology and isotope data and allowing both removal and adding of variables. For vegetative and wood cover we used the same procedure to fit a binomial GLM.

4.4 RESULTS

4.4.1 Assortative mating

There was significant assortative mating by trophic position lakewide (r_{m*e} = 0.282, P = 0.004, Figure 4.2A). For % benthic carbon, only the SE shore showed a significant correlation (r_{m*e} = 0.453, P = 0.039, Figure 4.3A). Egg isotopes were strongly correlated with female liver isotopes (Nitrogen: r_{f*e} = 0.799, $P < 0.0001$, Carbon: r_{f*e} = 0.976, $P < 0.0001$), supporting the use of eggs as a proxy for female liver isotopes. Using the correlation between female liver and egg isotopes we estimate correlation between

male and female trophic position is $\hat{r}_{m*f}$ = 0.353 (SE = 0.0513). The estimated correlation between male and female % benthic carbon (on the SE shore) is $\hat{r}_{m*f}$ = 0.464 (SE = 0.0966).

4.4.2 The role of spatial cosegregation in assortative mating

In univariate correlations, male trophic position was significantly correlated with shore, nest depth, and vegetation cover. Egg (female) trophic position was only significantly correlated with shore (table 4.1A). However, the observed assortative mating for trophic position (r_{m*e} = 0.282, 95% confidence interval: 0.091-0.452) is stronger than could be explained by spatial cosegregation of diet measures with any of the measured habitat features (table 4.1A). For example, cosegregation by shore only would lead to a r_{m*e} of 0.083 (see figure 4.4). Thus, spatial cosegregation of diet strategies can generate some weak assortative mating ($r < 0.1$), but is insufficient to explain the observed assortment.

The measured habitat variables are not independent, so we also carried out a test for assortative mating that accounted for multivariate microhabitat data. Both male and egg isotopes were separately modeled as functions of multiple habitat variables; if there is assortative mating above and beyond the effect of habitat, the residuals of these models should be correlated. In a linear model, male trophic position was significantly associated with nest depth (P = 0.0054) and shore (P = 0.00032; table 4.2A). Egg trophic position (a proxy for females) was also correlated with shore (P = 0.0038; table 4.2B). Both males and eggs had higher trophic position on the SE shore. Residuals of these models were calculated to control for patterns of association between diet and nest habitat and location. Significant male/egg correlations remained after accounting for these spatial

effects, as demonstrated by correlations between residual isotope scores (Trophic postion: $r = 0.232$, $P = 0.019$, Figure 4.2B).

Predicted correlations between male and female % benthic carbon due to univariate spatial cosegregation were all negative and outside the confidence interval of the correlation on the southeast shoreline ($r_{m*e} = 0.453$, 95% confidence interval: 0.027-0.740), except for depth (table 4.1B). Both males and females showed a negative but non-significant correlation between depth and % benthic carbon, with a predicted correlation between male and female diet of 0.0406 (table 4.1B). The partial correlation between male and female % benthic carbon and nest depth fell within the 95% confidence interval of the observed correlation (table 4.1B). It is worth noting, however, that the observed benthic carbon assortment on the southeast shoreline has wide confidence intervals due to small sample size on that shoreline. Because the estimate of r_{m*e} fell within the 95% confidence interval it is necessary to further explore whether nest depth alone explains the observed level of assortment. We found the residuals of the relationships between % benthic carbon and nest depth for male and eggs were significantly correlated ($r = 0.450$, $P = 0.041$), implying that assortment remains even after we remove the covariance between diet and nest depth. This result is consistent with our finding that the assortative mating expected from spatial co-segregation is less than we actually observe. We therefore conclude that none of the measured habitat variables can, by itself, account for the observed correlation between male and female % benthic carbon.

For % benthic carbon a multivariate model was made using only the nests located on the SE shore as this was the only location showing assortative mating by carbon isotopes (Table 4.3). No factors were significant in these models for male or female % benthic carbon. Significant male/egg correlations remained after removing spatial effects using residuals of these models (% benthic carbon on the SE shore: $r = 0.568$, $P = 0.007$,

Figure 4.3B).In addition, we performed post-hoc tests to explore why the pattern of assortment by % benthic carbon differed between the NW and SE shore. We used linear models of lakewide % benthic carbon for males and eggs with habitat variables, including a shore*habitat interaction term. We found that there is a significant shore*depth interaction for egg % benthic carbon (P= 0.048), with more benthic females tending to lay eggs shallower on the SE shore (where assortative mating was observed) and tending to lay eggs deeper on the NW shore. The significance of assortment on the SE shore relies heavily on the nest with the lowest % benthic carbon for both male and egg (Figure 4.3). This point has high leverage and a Cook's Distance > 1. Removal of this point makes the relationship between male and female % benthic carbon non-significant (% benthic carbon on the SE shore: $r = 0.192$, $P = 0.42$). However, careful examination of the data reveals no obvious bias or error in the collection of the isotope data and so it is inappropriate to remove this data point from our analysis. Note that this outlier does not influence the habitat-corrected assortative mating with respect to trophic position, described above.

4.4.3 Relationship between nest habitat and male diet and morphology

Based on AIC model selection, we found that males tended to have deeper nests when they exhibited more limnetic carbon, higher trophic position, and nested on the NW shore (table 4A). There was a marginal tendency for males with shorter residual gill raker length to nest deeper. Males with higher trophic position tended to nest in denser vegetation, and there was a marginal effect of gape width and shore (table 4B). Finally, larger males tend to nest closer to wood cover (table 4C). These results suggest that males with different diet and phenotype partition nest habitats more finely than previously

suspected, as the depth gradient over which these traits differ spans only 2 vertical meters. Thus, there are systematic ecological and morphological differences between fish that are often just a few horizontal meters apart.

4.5 DISCUSSION

Assortment is measured as a correlation between male and female traits, but like any correlation there is no implication that the focal trait actually causes the assortment (e.g., that individuals exhibit a preference for phenotypically similar mates). It is therefore important to disentangle the various processes that may generate assortative mating, in order to understand the causes of reproductive isolation among individuals, between populations, and the resulting genetic structure. Two major alternative processes are plausible – individuals may either tend to encounter similar phenotypes when searching for a mate (spatial or habitat isolation), or they may select similar phenotypes from among the pool of individuals that they encounter (mate preferences). We show that, at least in stickleback, phenotypic divergence across space and microhabitats does occur within even small lakes, and can generate weak assortative mating. However, although nest habitat and location are associated with diet and morphology, these associations are not sufficient to explain the level of assortative mating observed in this population. Our results therefore suggest that mate preference rather than spatial isolation is key to generating assortative mating by diet in this population. We explore the levels of assortative mating that can be created by cosegregation without direct mate preference and conclude that our results are likely quite general across taxa.

Spatial isolation of individuals with different diets could take two general forms. First, individuals found in isolated locations might feed on different prey and rarely

interbreed due to reduced encounter rates. In the context of our study, this could appear as across-lake spatial gradients in diets or isotope signatures which generate assortative mating because ecologically divergent individuals never encounter each other. We did find evidence that individuals nesting on different shores have significantly different isotope signatures. Whether we analyze the raw data ($\delta^{13}C$ and $\delta^{15}N$) or their spatially adjusted equivalents (% benthic carbon and trophic position), males and females differ in isotopes in the same direction between shores. Thus, some of the signal of assortative mating on a whole-lake level may be due to across-lake phenotypic differentiation. The causal basis of this differentiation is not clear. Are individuals nesting on different shores constrained to have different diets, or do individuals with different prey preferences opt to settle in different locations? The latter explanation seems more likely. If individuals randomly choose a shore to nest on and subsequently acquire different diets, males (which are constrained to remain near their nest) should exhibit greater between-shore isotope differences than females, which may move freely between shores. Quite the contrary, females exhibit greater between-shore isotope differences than the less mobile males. We therefore posit that individuals choose which shore they nest on based on already-established prey or habitat preferences. Bolnick et al (2009) previously showed that habitat preference can reduce stickleback dispersal between habitats, facilitating adaptive divergence.

A second general form of spatial isolation arises if the various phenotypes are spatially well mixed and frequently encounter each other, but prefer subtly different microhabitats when searching for mates. In the context of stickleback, this could represent differences in nest-site selection by males and females. In the benthic/limnetic stickleback species pairs and in benthic-like lakes and limnetic-like lakes, limnetic diet is associated with nesting in open, shallower areas and benthic diet with nesting in dense

vegetation at deeper depths within the littoral zone (McPhail 1994; Vines and Schluter 2006). Interestingly, we found the opposite trend for most traits: males and females with stereotypically 'limnetic' traits (lower % benthic carbon, higher trophic position, Matthews et al 2010; and smaller gape width, Robinson 2000) tended to nest or lay eggs in deeper nests. Also, males guarding nests in dense vegetation had smaller gapes (a limnetic trait) and higher trophic position. The one exception was that individuals with shorter gill raker length (a benthic trait; Robinson 2000) used deeper nests. The atypical results are not simply due to differences between shores, because the trends hold within a given shore. Nests tended to be deeper on the NW shore, where fish tended to have higher % benthic carbon and lower trophic position. Wood cover was only significantly correlated with male standard length, with larger males (a benthic trait) guarding nests that were shielded by large logs. Although this type of cover has not been analyzed in previous studies of nesting habitat, it should provide shelter in a manner similar to nesting among dense vegetation favored by larger (Kraak et al. 2000) or benthic males (McPhail 1994). In this population the associations between habitat and ecotype primarily go against *a priori* predictions derived from the benthic/limnetic species pairs.

Correlations between male and female isotopes generated by spatial cosegregation are too weak to explain the observed correlations between males and females. For nitrogen isotopes, all patterns of spatial cosegregation are too weak to explain the observed correlation between male and female trophic position. In contrast, carbon stable isotopes were correlated with depth for both sexes on the southeast shore. Based on this cosegregation, we calculated a predicted male-female correlation that was substantially less than the observed male-female correlation, but was within its 95% confidence interval. However, the confidence interval for this correlation is quite broad because of the small sample size on the SE shore. Analyses of isotope residuals provide clearer

evidence that cosegregation is insufficient to explain assortative carbon assortative mating. Individuals with more benthic isotope residuals (for a given nest depth) tend to mate with individuals with more benthic isotope residuals (also controlling for nest depth).

Despite being too weak to generate appreciable assortative mating, the fine-scale phenotype-environment correlations found here (e.g., over a depth gradient of approximately two meters), are themselves quite noteworthy. Most studies of habitat choice have dealt with either discrete habitats or discrete phenotypes, but this study suggests animals may partition habitats much more finely than typically appreciated. Such partitioning can generate biased encounter rates between phenotypes. Biased encounter rates are a major component of models of speciation by habitat isolation, such as is seen in host races of phytophagous insects. The role of spatial separation is quite obvious in such cases, where plant hosts represent discrete and distinct entities. However, phenotypes may also be distributed non-randomly across more subtle environmental gradients, as we document here for three-spine stickleback. If these fine-scale correlations between phenotypic traits and continuous environmental variables are very common, subtle micro-habitat differentiation might commonly play a small role in generating assortative mating.

The strength of assortative mating observed in this study was similar to that observed in our previous study (Chapter 3), and thus suggests that assortative mating by diet may be quite general in stickleback. In the previous study we used a principal component axis to summarize isotope signatures ($\delta^{13}C$ and $\delta^{15}N$ were correlated) and found a male-egg correlation of $r_{m*e} = 0.348$ and an estimated male-female correlation of $\hat{r}_{m*f} = 0.507$ (Chapter 3). In contrast, the present study examined each isotope separately, as the isotopes were uncorrelated. For trophic position the correlation between the

isotopes of male liver and eggs in their nest was r_{m*e} = 0.282. Given the female-egg correlation, we estimate that the strength of assortative mating (male-female correlation) is $\hat{r}_{m*f}$ = 0.353. For % benthic carbon assortative mating only occurred along one shore and the male-egg correlation was r_{m*e} = 0.453, yielding an estimated male-female correlation of $\hat{r}_{m*f}$ = 0.464. These numbers imply that diet-assortative mating may be slightly weaker than previously observed in Mohun Lake, though direct comparison is complicated by differences in the spatial extent of sampling and differences in C~N correlations between studies. These observed differences in the strength of assortment raise the intriguing possibility that assortative mating varies among populations, and the corresponding question of what ecological or evolutionary forces explain such variation in the strength of assortment.

The suggestion that assortment varies between populations is further supported by our finding of carbon-assortment on only one shore of Burnt-Out Lake. It is unclear why assortment by % benthic carbon only existed for one shore and showed no pattern on the other shore. There was a significant depth*shore interaction for egg % benthic carbon. On the SE shore, where carbon assortment was observed, both males and females tended to nest (or lay eggs) deeper when they had a more limnetic isotope signature. This mutual correlation between isotopes and depth could be due to spatial cosegregation, or because more limnetic females preferred to mate with more limnetic males, who happened to nest deeper. In contrast on the NW shore females showed a non-significant opposite trend, laying eggs shallower when they had a more limnetic isotope signature. Some caution is necessary in interpreting the difference in carbon assortment between shores because the significance of assortment on the SE shore is dependent on an outlier data point: a nest with low benthic carbon for both the male and female. However, there is no reason to believe the data from this nest represent anything beyond an assortative mating between a

male and female with unusual diet for their nesting area. It therefore is not proper to disregard this data in our conclusions. In fact, matings between individuals who are phenotypic outliers will be informative to our understanding of assortment as these individuals should have lower encounter rates with phenotypically similar individuals. Interestingly, rare phenotypes theoretically should become less choosy due to increased search costs (Real 1990; Crowley et al. 1991). Changes in choosiness due to differences in cost have been experimentally demonstrated in stickleback (Bakker and Milinski 1991; Milinski and Bakker 1992).

To generalize our results, it is helpful to ask how strong spatial segregation must be to generate plausible levels of assortative mating. Using standard principles of partial correlation, an indirect phenotypic correlation between males and females, via their joint association with a habitat variable, is equal to the product of the partial correlation between each sex's trait values and the habitat variable (Figure 4.4). Thus, assortative mating via spatial co-segregation requires both sexes to exhibit reasonably strong trait-environment correlations. Assortative mating for a given average trait-environment correlation is strongest when the two sexes show equal trait-environment correlations, and the strength of assortment will still be weaker than either trait-environment correlation alone. To illustrate this point, a recent meta-analysis of over 1,000 empirical estimates of male-female trait correlations within populations found an average strength of assortment of 0.28 (Jiang, Bolnick, and Kirkpatrick, manuscript). To achieve this typical level of assortment via co-segregation, both sexes must exhibit trait-environment correlations of 0.53. Such correlations are probably quite reasonable in some insect host races with discrete habitats (host plants), and when assortment arises from isolation by distance, but may be unusually strong for more subtle quantitative environmental variation within a single population, as studied here. Consequently we anticipate that the

results presented here for stickleback may be generally applicable to a wide range of species.

After controlling for the relationships between isotopes of both males and females with all measured microhabitat features and spatial differences, we find that there is a significant correlation between the residual variation. We therefore conclude that assortative mating with respect to both carbon source and trophic position are not merely the result of spatial structure of individual diet strategies during the breeding season. This conclusion must be accompanied with a caveat that it remains possible that males and females co-segregate along some unmeasured environmental gradient that reduces their encounter rates with different phenotypes. However, we measured the environmental variables most commonly seen to differ between benthic and limnetic species pairs (McPhail 1994; Vines and Schluter 2006) and our general results suggest that unmeasured environment-phenotype correlations must be quite strong in order to produce the strength of assortative mating observed here. Therefore, we feel fairly confident in concluding that while spatial structure may contribute weakly to assortative mating, there must be some active mate preferences generating diet-assortative mating.

This evidence for mate preferences is inferential, having been arrived at by process of elimination. However, there is some additional evidence supporting this conclusion. Most notably, individuals directly assess the diet of conspecifics (including potential mates) through olfaction. In one study, individual stickleback experimentally fed a particular prey type subsequently preferred to associate with shoals of individuals who have fed on that same prey (Ward et al. 2004). Olfactory cues were necessary and sufficient to generate such diet-assortative shoaling. Such associations could generate assortative mating in diet-variable natural populations. To test this possibility, we conducted a laboratory mate-choice study extending the results of Ward et al. (2004) to

the context of mating. Males and females were experimentally fed a particular either benthic or limnetic prey (chironomid larvae or Daphnia). Females were then used in mate-choice trails, choosing between unfamiliar same- and different-diet males, as well as no-choice mate trials. We found that gravid females associated preferentially with nesting males who had been fed the same experimental diet rather than a different diet than the female, when only olfactory cues were present (P= 0.009; Snowberg & Bolnick, unpublished data). Gravid females also progressed further through courtship with nesting males fed the same diet than a different diet (P= 0.017; Snowberg & Bolnick, unpublished data). This suggests gravid females may use diet cues directly in assessing potential mates, consistent with our inference based on the data presented here. In addition, olfactory cues are used in other contexts of stickleback mating, such as disassortative mating by MHC (Reusch et al. 2001) and assortment between benthic and limnetic stickleback species (Rafferty and Boughman 2006, Kozak et al. 2011). Another potential contributor to assortative mating in stickleback is phenotype matching of mates based on morphological traits that are correlated with diet. We are unable to measure a female's morphology using eggs collected from a nest and so this possibility would require observing matings or using other methods to find the female associated with each nest. These mechanisms of assortment are not mutually exclusive and may form a complex web of traits contributing to reproductive isolation.

In conclusion, we find that individuals can exhibit phenotype-environment correlations. When both males and females exhibit parallel phenotype-environment correlations (as occurs for some traits in stickleback), then positive assortative mating can result. However, we find that these phenotype-environment correlations must be quite strong in both sexes ($r > 0.5$) in order to generate weak assortative mating comparable to values typically seen within populations. We therefore suggest that spatial or habitat

segregation will be important in situations where trait-environment correlations are very strong, such as in insect host races, but may be less important in other settings. In either case, it will be generally important to control for both coarse spatial structure and fine-scaled habitat structure in future studies of assortative mating.

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A. Trophic Position: observed r_{m*e} = 0.282 (95% CI: 0.091-0.452)

Habitat Feature	r_{m*h}	P_{m*h}	r_{e*h}	P_{e*h}	r_{m*e}
Shore	0.267	0.007	0.309	0.002	0.083
Nest Depth	0.267	0.007	-0.132	0.19	-0.035
Vegetation Cover	0.241	0.015	0.089	0.37	0.021
Wood Cover	0.109	0.28	0.041	0.69	0.004

B. Percent Benthic Carbon: observed r_{m*e} = 0.453 (95% CI: 0.027-0.740)

Habitat Feature	r_{m*h}	P_{m*h}	r_{e*h}	P_{e*h}	r_{m*e}
Nest Depth	-0.112	0.63	-0.413	0.063	0.041
Vegetation Cover	0.167	0.47	-0.208	0.37	-0.034
Wood Cover	0.321	0.16	-0.199	0.39	-0.064

Table 4.1: Univariate correlations between diet and nest habitat for males (r_{m*h}) and eggs (r_{e*h}) and the predicted correlation between males and females (r_{m*e}) that would result from spatial cosegregation, calculated as $r_{m*h} * r_{e*h}$.

A. Males

	Estimate	Std. Error	t value	*P*
(Intercept)	2.972	0.0468	63.552	< 0.0001
Nest Depth	0.156	0.0547	2.850	0.0054
Vegetation Cover	0.0596	0.0328	1.814	0.073
Wood Cover	0.0412	0.034	1.189	0.24
Shore	0.139	0.0373	3.734	0.00032

B. Females

	Estimate	Std. Error	t value	*P*
(Intercept)	2.952	0.0570	51.809	< 0.0001
Nest Depth	-0.0783	0.0666	-1.175	0.24
Vegetation Cover	0.0647	0.0399	1.621	0.11
Wood Cover	0.0192	0.0416	0.461	0.65
Shore	0.135	0.0453	2.971	0.0038

Table 4.2: Model results of trophic position as a function of nest habitat. Correlations between the residuals of these models provide an estimate of assortative mating independent of habitat isolation.

A. Males

	Estimate	Std. Error	t value	*P*
(Intercept)	0.417	0.180	2.318	0.033
Nest Depth	-0.326	0.279	-1.169	0.26
Vegetation Cover	0.180	0.156	1.156	0.26
Wood Cover	0.209	0.137	1.533	0.14

B. Females

	Estimate	Std. Error	t value	*P*
(Intercept)	0.749	0.185	4.039	0.00085
Nest Depth	-0.449	0.287	-1.564	0.14
Vegetation Cover	-0.00273	0.160	-0.017	0.99
Wood Cover	-0.0975	0.141	-0.693	0.50

Table 4.3. Model results of % benthic carbon as a function of nest habitat for the southeast shore. Correlations between the residuals of these models provide an estimate of assortative mating independent of habitat isolation.

A. Nest depth

	Estimate	Std. Error	t value	*P*
(Intercept)	-0.870	0.554	-1.572	0.12
% Benthic Carbon	-0.191	0.0822	-2.328	0.022
Trophic Position	0.568	0.174	3.269	0.0015
Gape Width residual	-0.906	0.507	-1.786	0.077
Raker Length residual	-0.552	0.250	-2.206	0.030
Shore	-0.248	0.0669	-3.704	0.00036

B. Vegetative cover

	Estimate	Std. Error	z value	*P*
(Intercept)	-14.469	4.976	-2.908	0.0036
Trophic Position	4.468	1.574	2.838	0.0045
Gape Width residual	-7.803	4.278	-1.824	0.068
Shore	-1.080	0.590	-1.829	0.067

C. Wood cover

	Estimate	Std. Error	z value	*P*
(Intercept)	-7.812	3.041	-2.569	0.010
Standard Length	0.151	0.0686	2.207	0.027

Table 4.4: Best fit model results for nest habitat related to male isotopes and morphology. All models started with all factors included (% benthic carbon, trophic position, Standard Length, gape width residual, and gill raker length residual). All factors from the best fit model are included.

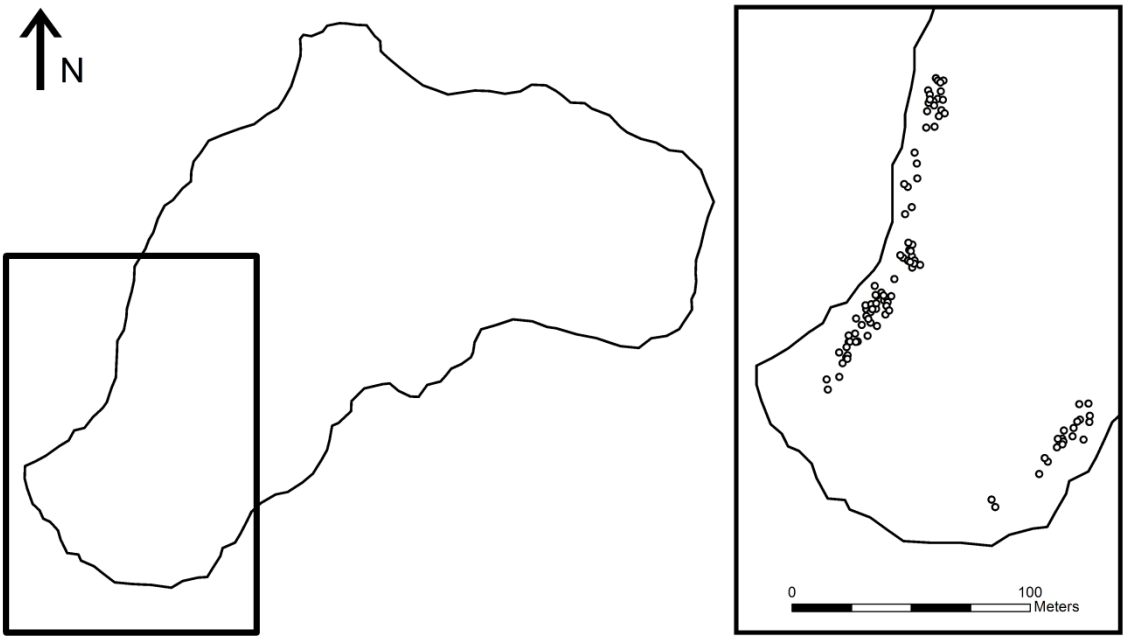

Figure 4.1: Stickleback nests in Burnt-Out Lake were located in two discrete nesting colonies on opposite shores. The area separating these colonies was searched for nests but consisted of marshy habitat and woody debris where stickleback did not appear to be nesting.

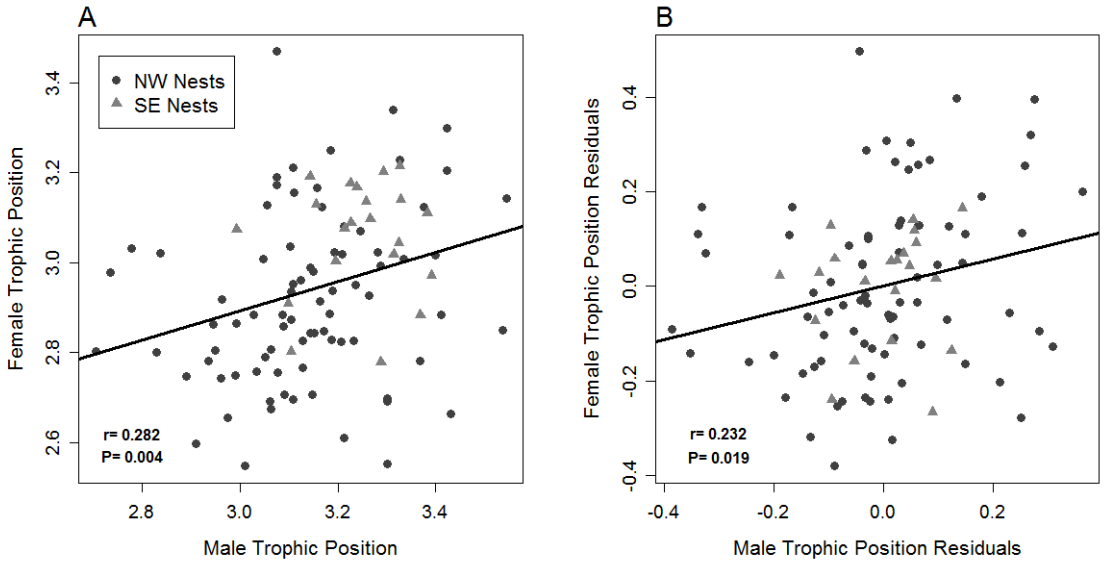

Figure 4.2: Male and female trophic position measured using male liver isotopes and female egg isotopes were significantly correlated (r_{m*e} = 0.282, P = 0.004; A). The residuals of the relationships between male and female isotopes with measured habitat variables were also significantly correlated (r = 0.232, P = 0.019) suggesting assortative mating is not simply explained by habitat (B).

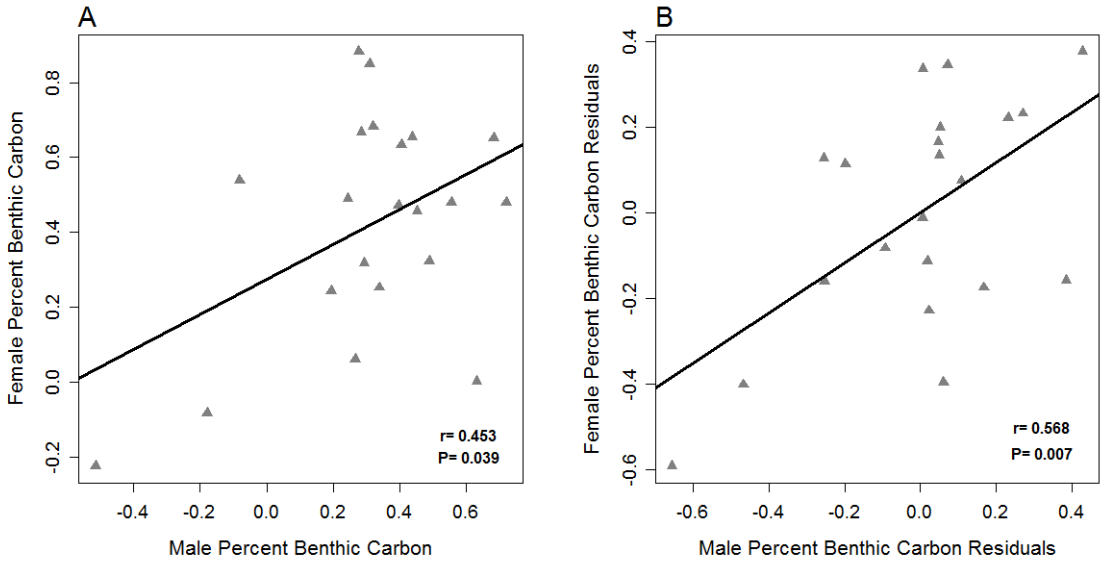

Figure 4.3: Male and female % benthic carbon measured using male liver isotopes and female egg isotopes were significantly correlated in nests collected on the southeast shore (r_{m*e} = 0.453, P = 0.039; A). The residuals of the relationships between male and female isotopes with measured habitat variables were also significantly correlated (r = 0.568, P = 0.007) suggesting assortative mating is not simply explained by habitat (B).

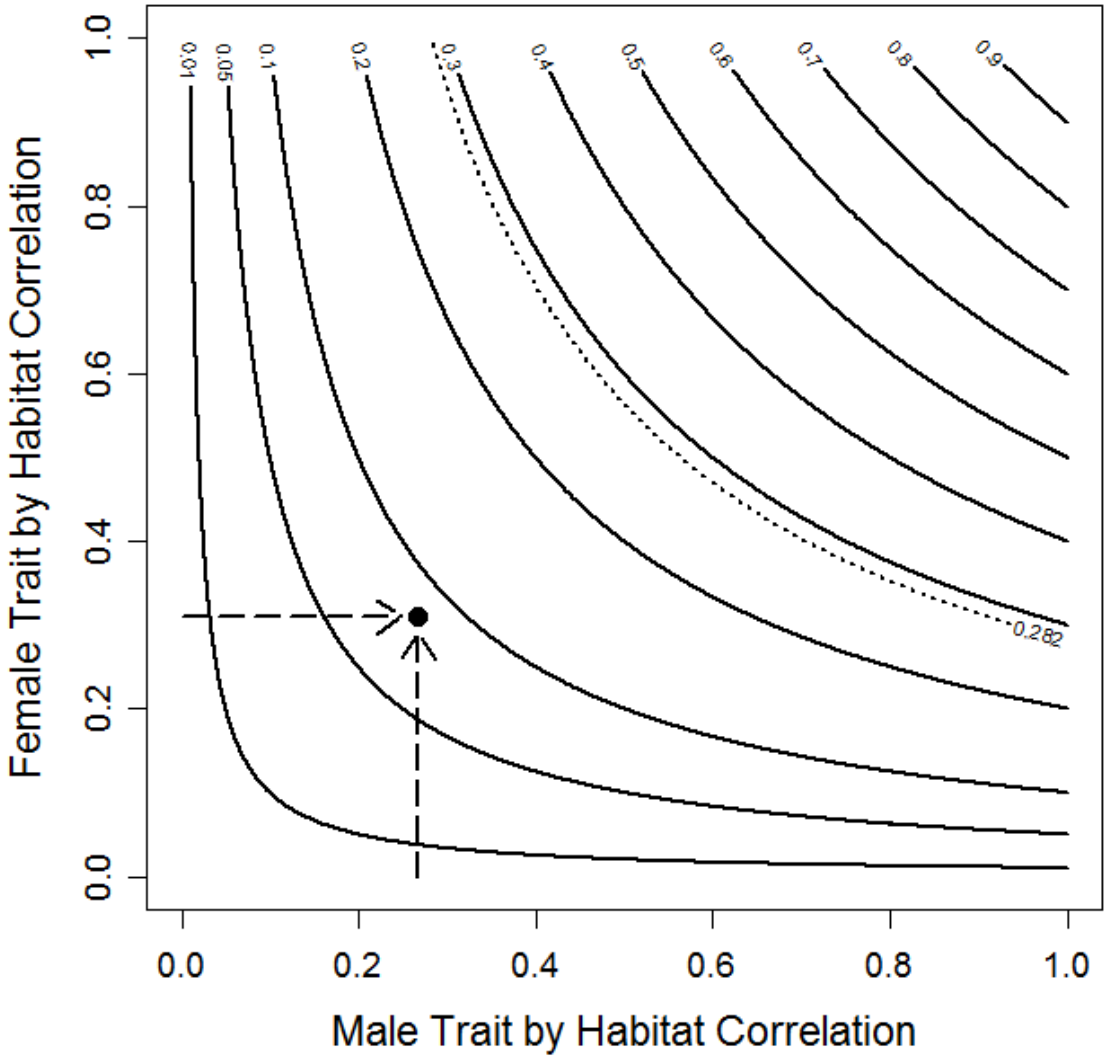

Figure 4.4: The strength of assortative mating via spatial co-segregation depends on the simultaneous strength of male-habitat and female-habitat correlations. The indirect partial correlation between males and females, r_{m*f} is the product of the partial correlations between each sex and a habitat variable (r_{m*h} and r_{f*h}), shown by contour lines. Dashed lines representative data from this study: the measured correlations between male and female tropic position and shore (both significant) lead to a predicted strength of assortment of 0.083. This value is significantly less than the measured correlation of 0.282.

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